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ABBREVIATIONS

aa	amino acid residues
ADCC	antibody-dependent cell-mediated cytotoxicity
BSA	bovine serum albumin
CD	cluster of differentiation
c.p.m.	counts per minute
EA-mIgG	erythrocytes sensitized with murine IgG
EA-mIgG2b	erythrocytes sensitized with murine IgG2b mAb
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FcγR	receptor for Fc moiety of IgG
FcϵR	receptor for Fc moiety of IgE
FcR	receptor for Fc moiety of immunoglobulin
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	unit of gravity
h	human
HLA	human histocompatibility leucocyte antigens
HR	high-responder
hr	hour
HRBC	human red blood cells
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin G
IFNγ	interferon-γ
IL	interleukin
kDa	kilodalton
LR	low-responder
m	murine
mAb	monoclonal antibody
MHC	major histocompatibility complex
mIgG2b	murine immunoglobulin G, isotype 2b
mIgG1	murine immunoglobulin G, isotype 1
min.	minutes
n	number in study or group
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SI	stimulation index
TcR	T cell receptor for antigen

Contents

Chapter 1

General introduction

The production of specific immunoglobulin (Ig) molecules (antibodies) is a central process in the immune response. Ig molecules exist in two different forms: as cell surface bound receptors that serve as antigen receptors present on B lymphocytes, or as proteins that are secreted by plasma cells to capture soluble or cell-bound antigens. Five classes of Ig molecules are characterized by specific antigenic determinants in their heavy chains: IgG (γ), IgM (μ), IgE (ϵ), IgA (α) and IgD (δ) (180). IgG molecules which are the prominent glycoproteins in serum are further divided in distinct subclasses; in man: human (h)IgG1, hIgG2, hIgG3 and hIgG4; in the mouse (m): mIgG1, mIgG2a, mIgG2b and mIgG3.

In addition to their specific binding to cell-bound or soluble antigen, the non-specific binding of IgG to human monocytes (63), human neutrophils (103) and to murine macrophages (81) was already described more than twenty-five years ago. Later, a similar binding to mouse B lymphocytes was observed by Paraskevas (112). From this latter study it appeared that the cells had affinity for the Fc portion of the immunoglobulins. On this basis the term "Fc receptor" was proposed for these binding sites (111). The Fc receptors (FcR) present on several types of cells are able to bind antigen-antibody complexes and to remove them from the body fluids. In this way, Fc receptors function as linkage molecules between the humoral and the cellular immune response.

In these early years binding of IgG or IgM by FcR was also observed on murine T cells and on human B cells. Binding of IgE was demonstrated on B cell suspensions contaminated with basophils; IgA or IgD binding was not reported (39). In the late 1970s, Fc receptors expressed on murine B cells were already found to be structurally different from the surface (s) Ig molecules, complement receptors, or MHC class II (H-2 Ir-gene associated) molecules; differences were found in molecular weight of the receptor molecules, in studies on inhibition of binding or (co-)capping of receptor molecules, and sensitivity to proteolytic enzymes like trypsin (39).

Due to several newly developed techniques that became available in biochemistry and immunology during the 1970s, research has extensively increased. The EA-rosetting technique which was already described in the late

1960s (90) appeared to be necessary in defining the presence of Fc-binding molecules. The technique is based on indicator erythrocytes (RBC) that are sensitized with Ig molecules of an appropriate isotype; subsequently, the sensitized RBC are incubated with different cell types whereafter the formation of rosettes proves the presence of Fc receptors on the type of cells under study (Figure 1). Later, this technique has been refined and extended (72) by using appropriate and well-defined (monoclonal) antibodies in combination with properly isolated and purified cells and cell suspensions.

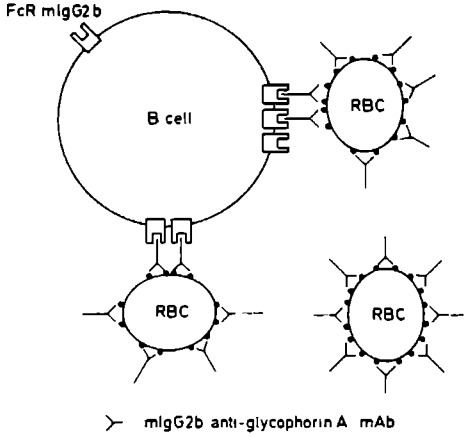

Figure 1.

EA-mIgG2b rosetting.

Human red blood cells (RBC) are sensitized with anti-glycophorin A mAb and are incubated with cells that express an appropriate FcR that is cross-reactive with murine immunoglobulin. In the present example, the RBC were sensitized with mIgG2b anti-glycophorin A mAb in order to study the FcR for mIgG2b. The percentage of EA-rosettes (cells which have bound at least three RBC) can be scored microscopically.

The further characterization of FcR for IgG (FcγR) and FcR for IgE (FcϵR) expressed on blood cell subsets and cell lines of human, murine or rat origin received much attention whereas FcR for IgM, IgA or IgD were hardly studied (32,95). In the latest decade, several isoforms of FcγR as well as of FcϵR, expressed on different hematopoietic cell types, have been well-characterized (also at the molecular level), and studies have been initiated with respect to their function.

CLASSIFICATION OF Fc RECEPTORS

Until now, three groups of Fc receptors have been defined (76,96). Nomenclature as proposed (128) will be used here. The first group consists of Fc receptors belonging to the immunoglobulin gene superfamily (66,187). This group includes Fc receptors that bind IgG with high-affinity (FcγRI) or low-affinity FcγR (FcγRII and FcγRIII), the high-affinity FcR for IgE (FcεRI) (13,104), the MHC class I-like FcγR which functions as an IgG transport molecule (12,145), and the polymeric IgA and IgM (poly-Ig) receptors (12,53). The second group of Fc receptors belongs to the calcium-dependent (C-type) animal lectin family (185), e.g. the low-affinity FcR for IgE (FcεRII; CD23). The third group of Fc receptors includes the FcR for IgA, IgM or IgD (22).

The investigations presented in this thesis deal with the interaction of human FcR with murine IgG2b mAb. These studies were performed with human monocytes and B cells. Since only FcγRI, FcγRII and FcεRII are present on resting monocytes and/or B cells (as illustrated in Figure 2) these Fc receptors will be discussed primarily.

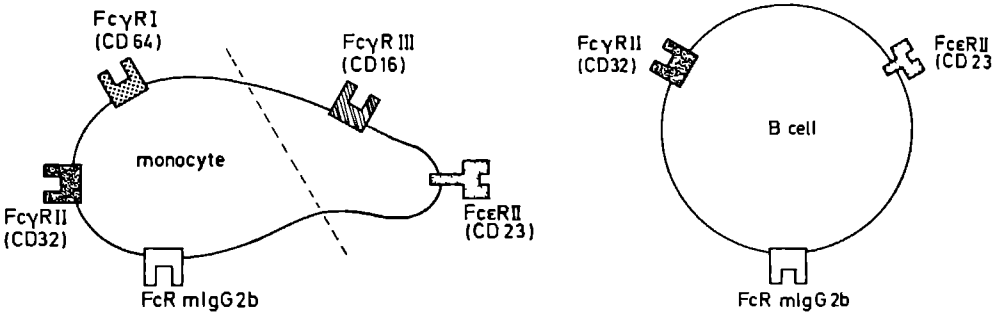

Figure 2. A B

Expression of Fc receptors on human monocytes (A) and B cells (B).

Constitutive expression of FcγRI, FcγRII and the FcR for mIgG2b is found on monocytes whereas FcεRII can be induced by IL4. FcγRIII is expressed on monocytes only after in vitro culture. On (EBV-transformed) B cells FcγRII, FcεRII and the FcR for mIgG2b are constitutively expressed.

HUMAN Fc RECEPTORS FOR IgG; hFcγR

History: In 1968, IgG binding to FcR-like structures on human cells was already described (63). In 1980, in humans the high-affinity FcR for IgG was first identified on myeloid cell lines U937 (4) and HL60 (34). On human myeloid cells (cell line U937) this FcR for IgG was shown to be trypsin-resistant (8), in contrast to its murine counterpart (163,165). Monomeric hIgG1 and hIgG3 were reported to bind to human mononuclear phagocytes (3,85). Almost simultaneously, different Fc receptors were isolated from human B and non-B cells (30,83). Further studies revealed the existence of two FcγR molecules with either high or low avidity for IgG on human macrophages (86) and on human myeloid cell line U937 (71). In 1986, on human monocytes these two FcR molecules were isolated and characterized by using affinity chromatography, immunoprecipitation with mAb (91), and differential ligand binding affinity (190). The presence of two FcR on human monocytes was also confirmed by anti-CD3 mAb induced T cell proliferation (29), EA-rosetting assays (172), modulation (173) and antibody-dependent cell mediated cytotoxicity (ADCC) (168).

One FcγR has high-affinity binding for human IgG, and can even bind monomeric hIgG1 and hIgG3. This receptor, a 72 kDa glycoprotein, is called hFcγRI. A second class of FcγR, hFcγRII, has a low-affinity binding, and can bind complexed hIgG1 and hIgG3. Its molecular weight is 40 kD. This Fc receptor for IgG on human monocytes and myeloid cell lines was proposed to be homologous to the low-affinity FcR for IgG on murine macrophages (166) identified by mAb 2.4G2 (126,164). On human monocytes, both hFcγRI and hFcγRII were found to be resistant to proteolytic enzymes. Remarkably, proteolysis strongly enhances the binding affinity of the human low-affinity hFcγR on monocytes (155,174). The hFcγRII was also detected on human platelets (132), neutrophils and eosinophils (93). Proteolytic treatment of human neutrophils demonstrated that the hFcγRII was resistant to leucocyte elastase (161). A third class of hFcγR, later defined as hFcγRIII (another low-affinity receptor) is present on human neutrophils, macrophages, natural killer (NK) cells and cultured monocytes (128,167). Immunoprecipitation of hFcγRIII reveals a broad band (50 - 80 kD), due to heterogeneity of glycosylation. On human neutrophils, the hFcγRIII is abundantly glycosylated and glycosylation

is important for ligand binding (74). Expression of hFcγRIII on neutrophils is sensitive to treatment with pronase or leucocyte elastase (50,161).

Cross-reactivity: All three hFcγR can be cross-reactive to some extent with murine immunoglobulins of different isotypes. hFcγRI can bind mIgG2a and mIgG3 antibodies (even in monomeric form), whereas hFcγRII can interact with mIgG1 sensitized cells (91,94) and perhaps with aggregated mIgG2b (71). hFcγRIII can also bind mIgG, primarily mIgG3, with low-affinity.

General functions: The general characteristics of the three different FcγR classes are presented in Tables 2, 3 and 4 (see below). Macrophages constitutively express all three FcγR receptor classes (75,128,167). On most cell types, expression of FcγR is regulated by cytokines. Cytokines can induce additive or antagonistic effects on the expression of FcγR (15). The three FcγR classes are involved in the mediation of calcium fluxes, phagocytosis, generation of superoxide, release of inflammatory mediators, and ADCC (169). Binding epitopes in the lower hinge region (CH2 domain) of IgG are essential in FcγR-mediated activation (22). The molecular structure of the hFcγR molecules was first identified during the late 1980s (2,137,150) and has been extensively reviewed in recent years (128,167,169).

The "Kurlander phenomenon": Several mAb against FcR (Table 1) have been described that are very useful in studying Fc receptors. Some of these mAb bind to the ligand binding site of the FcR and in this way block FcR functioning. Antibodies, however, can also block FcR by a different mechanism: the "Kurlander phenomenon". As was first described by Kurlander (84), the Fc moiety of an antibody that is specific for a cell surface antigen may also bind to an FcR expressed on the same cell, resulting in a tripartite binding of one IgG molecule to a single cell. Use of $F(ab')_2$ fragments of the IgG molecule can discriminate between the "Kurlander-type" of inhibition, or Fab-mediated inhibition.

In contrast to inhibition of FcR functions mediated by blockade of the ligand binding site via the Fab part of anti-FcγR mAb or via the "Kurlander phenomenon" via the Fc part, stimulatory effects on FcR functioning have been reported as well. A special case of the "Kurlander phenomenon" arises when

Table 1. **Characteristics of some anti-human Fc receptor mAb**

Antigen	mAb	Isotype	Blockade of ligand binding site	References
FcγRI	197	mIgG2a	yes (via Fc)	(60)
(CD64)	10.1	mIgG1	yes (via Fab)	(40)
	22	mIgG1	no	(60)
	32	mIgG1	no	(5)
	44	mIgG1	no	(107)
	62	mIgG1	no	(5)
FcγRII	CIKM5	mIgG1	yes (via Fc)	(159)
(CD32)	IV.3	mIgG2b	yes (via Fab)	(91,132)
	KB61	mIgG1	no	(125)
	41H.16[a]	mIgG2a	no	(11,56)
	2E1	mIgG2a	no	(47)
	KuFc79	mIgG2b	yes	(177,188)
	CIKM3	mIgM	no	(159)
FcγRIII	3G8	mIgG1	yes (via Fab)	(48,51,120,121)
(CD16)	B73.1	mIgG1	no	(48,117,121)
	CLBgran 1	mIgG2a	yes	(48,118)
	CLBgran 11[b]	mIgG2a	no	(48,65,118,159,162)
	GRM 1[b]	mIgG2a	no	(48,65,139,162)
	Leu 11a	mIgG1	no	(121,123)
FcεRII	Tü1	mIgG1	no	(18)
(CD23)	MHM6	mIgG1	yes	(18,133)
	25	mIgG1	yes	(18)

a) This antibody recognizes the polymorphism in hFcγRIIa.
b) These antibodies recognize the allelic forms hFcγRIIIb-NA1 (CLBgran 11) and hFcγRIIIb-NA2 (GRM 1).

a mAb directed against an FcR also binds by its Fc moiety. This antibody can then cross-link two FcR molecules without the use of second antibodies as bridging molecules, and activate the FcR-positive cells.

Until now, this mechanism was reported for two anti-FcγR mAb, mAb 197 (anti-hFcγRI; mIgG2a) and CIKM5 (anti-hFcγRII; mIgG1) (98,99,171). Triggering via hFcγRI by mAb 197 was measured in superoxide generation (122) and intracellular calcium responses (80,99,171); stimulation of hFcγRII by mAb CIKM5 was evident from intracellular calcium responses (97,98,171) and ADCC (80).

Functional polymorphism mediated by FcγR: The presence of Fc receptors in a mononuclear cell suspension has been analyzed in a T cell proliferation assay by using anti-CD3 mAb (156). The anti-CD3 mAb induced mitogenic response of T cells is supported by FcR-positive accessory cells and only occurs when the Fc part of the anti-CD3 mAb is able to interact with appropriate Fc receptors on the accessory cells (Figure 3).

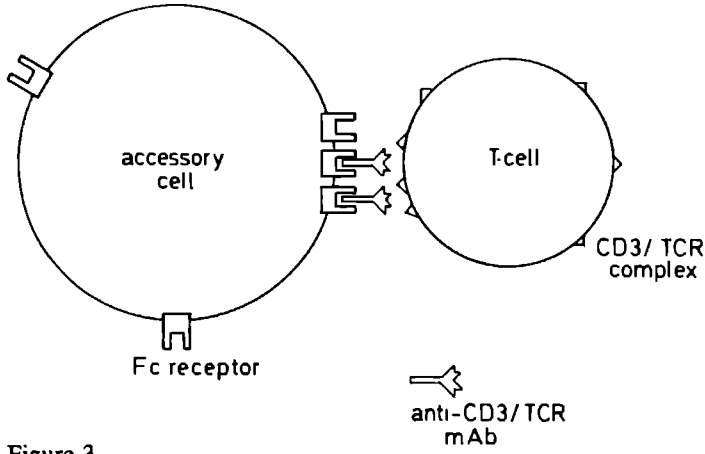

Figure 3.

T cell proliferation assay.

A mitogenic response of T cells induced by anti-CD3/T cell receptor (TcR) mAb requires the interaction of the Fc moiety of the antibody with an appropriate FcR on FcR-positive accessory cells. In this model T cell proliferation (measured by [^{3}H]-thymidine incorporation), therefore, provides an indirect measure of FcR function.

Table 2. **General characteristics of human FcγRI**

Cluster Differentiation	CD64
Protein (kDa)	FcγRIa: 72
Cellular distribution	monocytes/macrophages; neutrophils (after IFNγ); neutrophils (after G-CSF)
Polymorphism	Absence of receptor described in one family
Ligand binding and rankorder	hIgG: 3>1>4>>>2; mIgG: 2a=3>>>1=2b; rabbit IgG
Affinity for hIgG (Ka)	10^8 - 10^9 M^{-1}
Protein structure	single chain FcγRIa: 3 Ig-like domains FcγRIb2: 2 Ig-like domains
Signal transducing proteins	associated γγ-homodimer ?
Gene localization (chromosome)	1 q21.1
Number of genes; isoforms	3 genes; 4 isoforms
(TM: transmembrane receptor)	FcγRIa ; TM FcγRIb2 ; TM FcγRIb1 ; soluble FcγRIc ; soluble

Induction of a T cell proliferative (mitogenic) response by mIgG2a (or mIgG3) anti-CD3 mAb mediated via hFcγRI is measured in peripheral blood mononuclear cells (PBMC) from all individuals except four individuals from one Belgian family (24,169), whereas a proliferative response to mIgG1 anti-CD3 mAb via hFcγRII is measured in about 70 - 80% (High-Responders: mIgG1-HR) of the Caucasian, Negro and American Indian individuals tested (1,156,175); by contrast, in a population of Japanese individuals only 15% has been shown to be high-responder to mIgG1 anti-CD3 mAb (1). T cell activation mediated by mIgG2b anti-CD3 mAb occurs in about 10% of the Caucasian individuals (146,157,175).

Furthermore, a polymorphism in the neutrophil specific antigen system, NA1 and NA2 located on the neutrophilic hFcγRIII molecule is demonstrated by serology (87,159). The transmembrane hFcγRIII present on cultured monocytes/macrophages and NK cells is not polymorphic (41).

The human high-affinity Fc receptor for IgG; hFcγRI; CD64

General expression of hFcγRI: The human high-affinity receptor for IgG (Table 2) is constitutively expressed on monocytes, macrophages and myeloid cell lines like U937, HL60 and THP-1 (49). Expression of hFcγRI can be upregulated by IFNγ on cultured monocytes and phagocytes of alveolar (6) or placental (142) origin and induced on neutrophils in vitro (119,144) and in vivo (59). Increased hFcγRI expression on blood monocytes is induced by IFNγ (55,70), glucocorticoids (110) or a combination of these agents (55), and by IL10 (158). Additionally, IFNγ affects the cytolytic activity of cultured monocytes: treatment with IFNγ (9 days) perform hFcγRI-mediated ADCC whereas in the absence of additional stimuli long-term cultured monocytes mediate hFcγRI-induced phagocytosis. Monocytes cultured for a short period (40 hr) in the presence of IFNγ are able to mediate both functions (176).

By using anti-FcγRI mAb (Table 1) at least three epitopes on hFcγRI have been determined: the ligand binding site which is blocked by mAb 10.1; a second epitope detected by mAb 22 and 44; and a third epitope recognized by mAb 32 and 62 (46). On monocytes and U937 cells, hFcγRI has an additional binding site for C-reactive protein, an acute-phase protein (35).

Molecular structure of hFcγRI: The hFcγRI molecule possesses an extracellular region with three Ig-like domains and is encoded by the hFcγRIA gene (the hFcγRIa transcript) (2,170). Recently, an isoform encoded by the hFcγRIB gene, the hFcγRIb2 transcript, was isolated which has lost the coding sequence for the third domain that mediates the high-affinity binding of the receptor. The cytoplasmic tail is identical to that found in the hFcγRIa encoded molecules (45,124). Furthermore, two soluble products encoded by hFcγRIb1 and hFcγRIc transcripts have been isolated (45).

Signal transduction via hFcγRI: Stimulated expression and signal transduction via hFcγRI is induced by IFNγ and occurs on the transcriptional level. Recently, a DNA binding factor has been described (189) which recognizes the conserved motif of an IFNγ-response region on the hFcγRI gene (16,115). Single cell analysis of transfected cells with hFcγRIa isoform (wild-type) or deletion mutants expressing a transmembrane protein without a cytoplasmic tail (tail-minus) demonstrated that binding of IgG can be mediated by both receptor molecules whereas triggering of intracellular calcium and, subsequently, induction of phagocytosis can only be mediated by the hFcγRIa wild-type transfected cells (69). Cross-linking of hFcγRI molecules was recently reported to induce phosphorylation of phospholipase C-γ1 (PLC-γ1) via cytoplasmic tyrosine kinases functionally coupled to the Fc receptor (89).

Furthermore, in cultured macrophages hFcR-mediated (and also complement receptor CR1- and CR3-mediated) phagocytosis, triggered by protein kinase C, require intact actin filament assembly of the cellular cytoskeleton. With respect to hFcγRI (not hFcγRII or hFcγRIII) a direct intracellular association with an actin-binding protein has been reported. The actin-binding protein, a homodimer of 280 kDa is a cross-linking protein of actin filaments and is involved in stabilizing the cellular membrane. Extracellular binding of IgG molecules to hFcγRI disrupts (in a dose-dependent way) the intracellular complex of actin binding protein-hFcγRI immediately (within one minute) (109). Similar results have been found with respect to the murine FcγRIIβ2 expressed on macrophages: binding of immune complexes reduces the association of mFcγRIIβ2 and cytoskeletal proteins and results in impaired phagocytosis of these cells (152). In this context it is interesting to speculate on the physiological role of hFcγRI in vivo. Due to high concentrations ($\pm 10^{-5}$ M) of hIgG in plasma and the high-affinity binding of the receptor, hFcγRI is likely to be saturated with (monomeric) hIgG and, thus, intracellularly dissociated

from the actin binding protein. As a result, hFcγRI-mediated phagocytosis might also be reduced in vivo.

The human low-affinity Fc receptor for IgG; hFcγRII; CD32

General expression of hFcγRII: The hFcγRII (Table 3) has a broad cellular distribution and is expressed on monocytes and macrophages, neutrophils, B cells, eosinophils, and platelets (167). Furthermore, the human low-affinity hFcγRII is also present on epidermal Langerhans cells (140,141), human basophils (10), Schwann cells and perineurial cells (178), and on several cell lines such as the proerythro-myeloid cell line K562 (25,67) and the myeloid cell lines U937 and HL60 (101). In vitro, release of hFcγRII from blood lymphocytes (102) and activated B cells (135,136) has been found whereas, in vivo, release of soluble hFcγRII in serum has been reported in patients on hemodialysis and after renal transplantation (68).

General functions of hFcγRII: Freshly isolated hFcγRII-positive neutrophils and eosinophils have been reported to be unresponsive in initiating hFcγRII-mediated ADCC (57) or superoxide production (131). By contrast, freshly isolated monocytes are able to mediate ADCC of sensitized human erythrocytes (168). Cross-linking of proteins encoded by hFcγRIIa transcripts or by hFcγRIIc (that encodes a cytoplasmic region identical to hFcγRIIa but different from hFcγRIIb1 or hFcγRIIb2) results in an increased intracellular calcium response (79,131). Furthermore, treatment of monocytes with IFNγ and/or the synthetic glucocorticoid dexamethasone does not cause modulation of hFcγRII mRNA or protein whereas on U937 cells hFcγRII is upregulated by IFNγ (31). In addition, cytokines like IL4 might also be involved in differential functioning of monocyte hFcγR, e.g. IL4 downregulates hFcγRI-mediated phagocytosis and also diminishes hFcγRII-mediated superoxide production (15). Therefore, regulation of monocyte functioning might be due to balanced cytokine-mediated pathways. In addition, effector cells like tonsil-derived B cells, EBV-B cells or B cell lines like Daudi that express hFcγRII isoforms are unable to perform hFcγRII-mediated killing even when cells have been preincubated with a cytokine-cocktail of IL2, IL4, IL6 and IFNγ (100). These data indicate that hFcγRII-mediated functions are dependent on isoform and cell type.

Table 3. **General characteristics of human FcγRII**

Cluster Differentiation	CD32
Protein (kDa)	FcγRIIa: 40 FcγRIIb: 40 FcγRIIc: 40
Cellular distribution	monocytes/macrophages; neutrophils; B cells; platelets; eosinophils; basophils; Langerhans cells; syncytiotrophoblasts; placental phagocytes and endothelial cells
Polymorphism	FcγRIIA: 70% mIgG1-HR/ hIgG2-LR

Ligand binding and rankorder	hIgG: 3>1=2>>>4;
	mIgG: 1>2b>>>2a=3;
	rabbit IgG; bovine IgG1;

Affinity for hIgG (Ka)	10^6 M^{-1}
Protein structure	single chain 2 Ig-like domains
Signal transducing proteins	unknown
Gene localization (chromosome)	1 q23-24
Number of genes; isoforms	3 genes; 7 isoforms
(TM: transmembrane receptor)	FcγRIIa1-HR ; TM FcγRIIa1-LR ; TM FcγRIIb1 ; TM FcγRIIb2 ; TM FcγRIIb3 ; TM FcγRIIc ; TM sFcγRIIa2 ; soluble

Polymorphism in hFcγRII: A functional polymorphism has been detected initially in the mIgG1 anti-CD3 mAb induced T cell proliferation assay (Figure 3) in which peripheral human blood monocytes function as accessory cells, defining high- (HR) and low-responder (LR) individuals to mIgG1 (70% and 30%, respectively) (29,154,156). This polymorphism in hFcγRII is also evident in platelets (92) and neutrophils (17). Three phenotypes could be detected biochemically in isoelectric focusing of hFcγRII suggesting codominant expression of two hFcγRII alleles (7). The polymorphism is additionally reflected in binding of mIgG1 sensitized human red blood cells (EA-mIgG1 rosetting) (172), immunofluorescence with anti-FcγRII mAb 41H.16 (56), ADCC against mIgG1-coated target cells (20) and release of IL2, IFNγ (52), TNFα (36) and IL6 (82) after binding of mIgG1 antibodies to hFcγRII. Moreover, in mIgG1-HR individuals high-affinity binding of mIgG1 is associated with low-affinity binding of hIgG2, and vice versa (183). The high-affinity binding of hIgG2 to the hFcγRII-LR isoform is confirmed in functional studies and is present on monocytes, neutrophils and platelets (113,114).

Molecular structure of hFcγRII: Due to FcR-gene duplication, recombination and mutation several structurally related low-affinity FcγR genes have evolved (127), and differential RNA splicing events result in multiple FcγR isoforms. Until now, three genes (hFcγRIIA, B and C) have been characterized encoding hFcγRIIa1, hFcγRIIa2, hFcγRIIb1, hFcγRIIb2, hFcγRIIb3 and hFcγRIIc transcripts (23,169). The genomic organization of the hFcγRII gene, that is located on chromosome 1 (127,134), shows structural homology with HLA class I and II genes (43) encoding Ig-like domains as observed in other members of the immunoglobulin gene superfamily.

The extracellular domains of the hFcγRIIa1 and hFcγRIIb (b1, b2 or b3) encoded proteins differ in 7 amino acid (aa) residues (149). The hFcγRIIa1 isoform expressed on monocytes and myeloid cells and its functioning have been reported previously as low-affinity hFcγR (71,91); this isoform is also detected on neutrophils, platelets, human placental cells and cell lines U937, HL60 and K562 (21,61,149). The mIgG1-polymorphism that has been described is defined by substitution of only one aa at position 131 of hFcγRIIa1 (61). Polymorphism of hFcγRIIa1 at position 27 has also been described (153,182) but only the residue at position 131 (Arg in mIgG1-HR, His in mIgG1-LR) is involved in the functional polymorphism (26,27,153,181).

The hFcγRIIb2 transcript (21,42,143,184) is present in human B cells, cultured monocytes, neutrophils and placental trophoblasts and cell lines U937, Daudi and Raji (but not K562). The isolated hFcγRIIb2 transcript has >95% homology with hFcγRIIa1 and encodes three possible N-linked glycosylation sites (21,42,143). Furthermore, the hFcγRIIb1 and hFcγRIIb2 encoded proteins have similar cytoplasmic regions except for a 19 aa-encoded insert in hFcγRIIb1 (21,184). Differential functions related to this insert have not yet been defined but have been studied in detail in the murine system. The mFcγRIIb1 (mainly on B lymphoid cells) and mFcγRIIb2 (mainly on macrophages) encoded proteins are identical except for a 47 aa insert in the cytoplasmic region of mFcγRIIb1. Functional studies on transfected cells have revealed that mFcγRIIb1 is involved in binding of immune complexes, capping, and intracellularly binding of actin whereas mFcγRIIb2 mediates endocytosis of antigen-antibody complexes, internalization via coated pits, antigen presentation, phagocytosis of IgG-coated particles, and ADCC (53,54,106). A conserved region of at least 10 aa is disrupted by the insert in mFcγRIIb1 and this actively prevents endocytosis via coated pit localization (105).

The hFcγRIIC gene (previously also named hFcγRIIa') has possibly evolved from a cross-over event of the hFcγRIIA and hFcγRIIB gene at an exon/intron break. Exons of the B gene encode the extracellular, the transmembrane region and the first 8 - 10 amino acids of the cytoplasmic region (>99% identity) (21,44,149) whereas the remaining part of the cytoplasmic region of hFcγRIIC gene product is encoded by exons from the A gene (>95% identity). The protein is expressed on placental syncytiothrophoblasts in high amounts and also on monocytes, B cells, neutrophils and cell lines U937, Daudi and Raji, but not on human T cells or cell line K562 (21,149).

Signal transduction via hFcγRII: Binding studies on the hFcγRIIa1 encoded protein revealed that binding of mIgG1 or anti-FcγRII mAb IV.3 or KuFc79 does not require extracellularly N-linked glycosylation or the presence of a cytoplasmic domain (116). However, two tyrosine residues within the cytoplasmic domain encoded by hFcγRIIa1 transcripts are essential in mediating calcium responses, cytolytic activity and phagocytosis (79,108). In platelets, cross-linking of hFcγRIIa1 initiates calcium responses (9) and also tyrosine phosphorylation (via src-related tyrosine kinases) of hFcγRIIa1 itself (62).

Binding studies on cells transfected with hFcγRIIb1 revealed that anti-CD32

mAb KB61, KuFc79, 41H.16 and AT10 (58) bind to these cells whereas no binding is observed with anti-CD32 mAb IV.3, 2E1, CIKM3 or CIKM5. Furthermore, EA-rosetting with mIgG1, mIgG2a and mIgG2b sensitized RBC (Figure 1) occurs when rosetting is performed at 37 °C. In addition, mIgG and hIgG of all isotypes except mIgG3 and hIgG2 are able to induce anti-CD3 mAb induced T cell proliferation in the presence of hFcγRIIb1-positive accessory cells; this response is completely inhibited by mAb 41H.16 (184). With respect to data obtained with transfected cells, however, one should keep in mind that very high numbers of FcR are expressed on these cells. Differences in ligand affinity can be also be obscured when very high amounts of mIgG1 anti-CD3 mAb (29) or erythrocytes highly sensitized with mIgG1 mAb (153) are used in functional studies.

Signal transduction mediated by hFcγRIIA, B and C gene encoded receptors has not yet been studied as extensively as in the murine system, but the conserved region of 10 aa that encodes endocytosis and formation of coated pits in mFcγRIIb2 is also present in hFcγRIIB gene products (21,54,105). Further investigations on functions mediated by hFcγRII isoforms expressed on different types of cells are required to elucidate the specific intracellular transducing pathways.

The human low-affinity Fc receptor for IgG; hFcγRIII; CD16

General expression of hFcγRIII: The human low-affinity FcγRIII (Table 4) is mainly expressed on macrophages and neutrophils (but not on B cells) and is the only FcR expressed on NK cells (128,167). Soluble hFcγRIII molecules released from neutrophils are detectable at a high concentration in human plasma (64). No expression of hFcγRIII is measured on normal monocytes but this FcR can be induced by incubation during a few hours with transforming growth factor-beta (TGFβ) (186). The hFcγRIII is also expressed on monocytes that have been cultured in vitro for several days (28), and on macrophages. Since hFcγRIII isoforms are absent from the cells (normal monocytes and EBV-B cells) studied in this thesis no further introduction on this receptor will be given. The molecular heterogeneity and biological functioning of the hFcγRIII has recently been reviewed (169).

Table 4. **General characteristics of human FcγRIII**

Cluster Differentiation	CD16
Protein (kDa)	FcγRIIIa: 67 FcγRIIIb-NA1NA1: 50 - 65 FcγRIIIb-NA2NA2: 65 - 80
Cellular distribution	neutrophils: FcγRIIIb (TNF-α↓); monocytes: FcγRIIIa (after TGFβ); NK cells and macrophages: FcγRIIIa
Polymorphism	FcγRIIIb-NA1 isoform: 46% FcγRIIIb-NA2 isoform: 88%
Ligand binding and rankorder	hIgG: 1=3>>>2=4; mIgG: 3>2a>2b>>>1; rabbit IgG
Affinity for hIgG (Ka)	10^6 M^{-1}
Protein structure	single chain 2 Ig-like domains
Signal transducing proteins	FcγRIIIa associated γ- and/or ζ-chains
Gene localization (chromosome)	FcγRIIIA: 1 q23-24 FcγRIIIB: 1 q23-24 γ- and ζ-chain: 1 q
Number of genes; isoforms	1 gene; 3 isoforms
(TM: transmembrane receptor)	FcγRIIIa ; TM FcγRIIIb ; GPI-linked

HUMAN Fc RECEPTORS FOR IgE; hFcεR

Two FcεR molecules have been reported that differ in their cellular distribution and binding affinity. The high-affinity FcR for IgE (FcεRI) found on basophils is trypsin-resistant (147) whereas a low-affinity FcR for IgE (FcεRII) (Table 5) is expressed on lymphocytes and macrophages, and appears to be trypsin-sensitive (8,147). Because of the expression of hFcεRII on monocytes and B cells, this type of Fc receptor will be discussed in more detail. The hFcεRII also appears of interest since a complete blockade of functioning of the FcR for mIgG2b by HLA class II molecules is described in this thesis, and an association of hFcεRII and HLA-DR antigens has been reported (19).

The human low-affinity Fc receptor for IgE; hFcεRII; CD23

General expression of hFcεRII: The human FcεRII/CD23 is constitutively expressed on B cells and inducible on monocytes, macrophages, eosinophils, platelets, T cells and Langerhans cells (32,38). The hFcεRII is involved (in a cell type dependent way) in regulation of IgE synthesis and B cell development, phagocytosis of IgE-coated particles, IgE-dependent cytotoxicity against parasites, and release of leukotrienes and other inflammatory mediators (37,191). Furthermore, FcεRII was described to be identical to the B cell differentiation antigen, CD23 (18,194).

General structure in relation to other FcR molecules: In human and mice, surface (s) $IgM^+/sIgD^+$ mature B cells express the FcεRII molecule which is lost after isotype switching to sIgG, sIgA or sIgE (73). The FcεRII consists of a single chain glycoprotein anchored in the cellular membrane by a single transmembrane region in an unusual membrane orientation: the COOH-terminus of the protein is oriented extracellularly whereas the NH_2-terminus is located intracellularly. Preliminary studies on molecular structure have demonstrated that some members of the C-type lectin family including FcεRII exist as dimers at the cell surface (14). In contrast to the carbohydrate-lectin interaction suggested by the homology to the lectin-family, ligand-receptor interactions (IgE

and FcεRII, respectively) do not involve carbohydrates since deglycosylated IgE binds well to the FcεRII (129,179).

Table 5. **General characteristics of human FcεRII**

Cluster Differentiation	CD23
Protein (kDa)	45
Cellular distribution	FcεRIIa: B cells; FcεRIIb: after IL4 on B cells, monocytes and eosinophils
Polymorphism	no
Ligand binding and rankorder	hIgE mIgE
Affinity for hIgE (Ka)	10^6 M^{-1}
Protein structure	single chain lectin-like domain
Signal transducing proteins	unknown
Gene localization (chromosome)	19
Number of genes; isoforms	1 gene; 2 isoforms: A en B
(TM: transmembrane receptor)	FcεRIIa ; TM FcεRIIb ; TM

Molecular structure of hFcεRII: The molecular structure of the lymphocyte hFcεRII was first elucidated by using cDNA cloning techniques and studies on CD23-transfected eukaryotic cells. Two isoforms from the single copy gene of hFcεRII have been described (32,38). The hFcεRIIa encoded protein is constitutively expressed on B cells whereas the hFcεRIIb encoded molecule is present on peripheral blood lymphocytes from atopic patients and inducible by IL4 on normal B cells, monocytes, and eosinophils (192). In addition, in IL4-treated B cells (38) the expression of hFcεRIIb is downregulated by IFNγ within a short time (one hour) via the inhibition of newly synthesized CD23 proteins (88). Regulation by IFNγ of monocyte hFcεRIIb expression is still controversial (130).

Signal transduction via hFcεRII: The hFcεRII proteins were demonstrated to be similar in their extracellular regions and an internal cleavage site sensitive to trypsin-like proteases has been observed (38). However, the intracellular domains of the hFcεRIIa and hFcεRIIb isoforms vary at 7 aa which causes functional differences in signal transduction (192). The hFcεRIIa isoform encodes a tyrosine residue (instead of a serine residue in FcεRIIb) which is required for FcεRIIa-mediated endocytosis of soluble IgE-complexes in B cells. Additionally, a tyrosine kinase of the src-family was found to be physically associated with hFcεRIIa in activated B cells (151). Furthermore, the hFcεRIIb isoform encodes an asparagine and a proline residue near the N-terminus which are critical in phagocytosis of IgE-coated particles (38,193). Association of hFcεRII with signal transducing molecules has not yet been reported.

AIM OF THIS STUDY

In the early 1980s monoclonal antibodies were introduced in the clinic: as immunosuppressive agents, e.g. anti-CD3 mAb, in the treatment of renal allograft rejection (33), and for tumor specific cancer therapy (148). However, despite the immunosuppressive effect of anti-CD3 mAb, these (murine) antibodies caused severe side effects such as fever, headache, nausea and diarrhoea (78,160) which later appeared to be related to interactions between the Fc part of the murine Ig molecule and human Fc receptors. In vitro studies showed that in ADCC mediated by macrophages or killer (K) cells, mIgG2a mAb seemed to be most effective whereas less activity was observed with mIgG2b or mIgG1 mAb (77,148). Later studies revealed that in man, mIgG2a mAb predominantly interact with the high-affinity hFcγRI whereas mIgG1 mAb cross-reacts with the polymorphic hFcγRII as described above. Much less information was available with respect to cross-reactivity of mIgG2b antibodies with human Fc receptors.

In the mouse, binding of mIgG2b antibodies is mediated by the low-affinity FcR, mFcγRII and mFcγRIII (128), whereas hFcγRII was shown to bind mIgG2b antibodies under certain conditions. Binding of aggregated mIgG2b is enhanced at low ionic strength, is inhibited by anti-FcγRII mAb, and is trypsin-resistant (71). Further evidence for the presence of a human FcR that interacts with mIgG2b antibodies was found with T cell proliferation assays. Similar to the polymorphism described for mIgG1, mIgG2b anti-CD3 mAb-mediated polymorphic responses of human PBMC have been reported that were independent from the mIgG1 polymorphism (157); later, this observation has been confirmed by other investigators (138,146,175). Therefore, the interaction of mIgG2b with human FcR-positive cells on PBMC apparently does not involve the ligand binding site for mIgG1. During several years, the low prevalence of mIgG2b-HR individuals has impeded further studies. Nevertheless, characterization at the cellular and functional level of the human Fc receptor cross-reactive with mIgG2b antibodies is required when mIgG2b mAb are to be used immunotherapeutically.

The aim of the present study was to investigate the (polymorphic) interaction of human Fc receptors with murine mIgG2b antibodies, and to compare the mIgG2b-related polymorphism with the previously described polymorphism with respect to binding of mIgG1. More specifically, the following questions were studied:

- Which human blood cells are able to support the T cell proliferation induced by mIgG2b anti-CD3 mAb?

- Is the polymorphism in the binding of mIgG2b mediated by hFcγRII?

- Is hFcϵRII involved in binding mIgG2b immunoglobulins?

- Do HLA class II molecules affect functions mediated by the FcR for mIgG2b?

- Does proteolytic treatment affect the binding of mIgG2b to the FcR for mIgG2b?

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Removal of monocytes from cell suspensions with anti-CD14 antibody and carbonyl-iron, using FcγR-dependent accessory function as a sensitive measure of monocyte presence

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ABSTRACT

Human (FcγRI-positive) monocytes are required as accessory cells when T cell proliferation is induced by murine IgG2a anti-CD3 monoclonal antibodies (mAb). This T cell proliferation assay provides a sensitive method for detecting the presence of monocytes (less than 1% of monocytes can be detected), and we have used it to monitor the effectiveness of different procedures for the removal of monocytes from peripheral blood mononuclear cells. Counterflow centrifugation, phagocytosis of carbonyl-iron, adherence to plastic, monocyte depletion with magnetic beads (Dynabeads M450), and panning with anti-CD14 antibodies, each strongly reduced the number of monocytes. However, none of these methods, when used on their own, were capable of completely abolishing the mitogenic response to murine IgG2a anti-CD3 mAb. A virtually complete depletion of monocytes was obtained when the panning procedure using anti-CD14 antibodies was combined with phagocytosis of carbonyl-iron. Importantly, this method could also be used with cryopreserved cells.

We have applied this improved method for the removal of monocytes, to study T cell proliferation induced by murine IgG2b anti-CD3 mAb. We were able to demonstrate with this model that cells other than monocytes were able to provide accessory function.

INTRODUCTION

Many methods are available to remove most monocytes from a suspension of peripheral blood mononuclear cells, e.g. adherence (11), panning using anti-monocyte mAb (4) or human gamma globulin (5), counterflow centrifugation (6), phagocytosis of carbonyl-iron (5), or elimination with magnetic beads (e.g. Dynabeads M450). It has been noted before, however, that it is very difficult to deplete monocytes to such an extent that the mitogenic effect of anti-CD3 mAb, which is dependent on monocyte Fc receptors (14,15), is completely abolished. The presence of only a few monocytes is sufficient to provide the accessory function in this T cell proliferation assay. It is therefore a very sensitive method for measuring small contaminations with accessory cells, the presence of less than 1% of FcγRI-positive cells (i.e., monocytes) being detectable. In a recent study we have described an FcγR for mouse IgG2b, which is polymorphic and present on human mononuclear cells (10). In order to study whether cells other than monocytes are able to provide accessory function for T cell proliferation induced by mIgG2b anti-CD3 mAb, we needed a method for the complete removal of monocytes from (cryo-preserved) mononuclear cells. None of the methods mentioned above was, in our hands, capable of depleting monocytes to such an extent that the FcγRI-dependent mitogenic effect of mIgG2a anti-CD3 mAb was completely abolished. Therefore, we developed a new depletion technique which is based on the monocyte-specificity of anti-CD14 mAb. CD14 is a 55 kDa glycoprotein, anchored by phosphatidylinositol (PI) to the surface of monocytes and macrophages (8,13). We now demonstrate that, using panning with WT14 (anti-CD14) in combination with phagocytosis of carbonyl-iron, we were able to reproducibly obtain a complete depletion of monocytes even when cryopreserved mononuclear cells were used.

MATERIALS AND METHODS

Cells: Peripheral blood mononuclear cells (PBMC) were obtained from buffycoats of healthy donors by centrifugation on a Ficoll-Hypaque gradient (density 1.077 g/ml, Pharmacia, Sweden). PBMC were used directly or were cryopreserved and used later. The recovery and viability of the cryopreserved cells were >70% and >95%, respectively.

Monoclonal antibodies: The following anti-CD14 mAb were used for the panning of monocytes: 63D3 (hybridoma cell line obtained from American Type Culture Collection, Rockville, MD); FMC17, Seralab, Sussex, UK; AML 2-23, Medarex, West-Lebanon, NH; and WT14, produced in our laboratory (3). Using a sandwich-ELISA procedure based on a technique described by Bazil et al. (1), we were able to demonstrate that these four anti-CD14 mAbs recognize four different and independent epitopes on the CD14 antigen (manuscript in preparation). In addition, anti-CD14 mAb anti-Leu-M3 (Becton Dickinson, Mountain View, CA) and mAb 197 (directed against CD64, the Fcγ receptor type I), that was obtained from Medarex, were used in immunofluorescence studies.

Elimination of monocytes: Several procedures were tested for eliminating monocytes from a freshly isolated or cryopreserved mononuclear cell suspension.

- Counterflow centrifugation: A lymphocyte-enriched (monocyte-depleted) cell suspension was obtained from PBMC by counterflow centrifugation (6). Cells were cryopreserved, and used later in the T cell proliferation assay.

- Phagocytosis of carbonyl-iron: PBMC were suspended in culture medium (RPMI-FCS) which consisted of RPMI-1640 medium (Gibco BRL) containing both Hepes and sodium bicarbonate, supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, 50 μg/ml streptomycin and 50 IU/ml penicillin. PBMC (at 5 - 10 x 10^6 cells/ml) were then incubated with 40 mg/ml carbonyl-iron powder at 37 °C for 1 hr while thoroughly resuspending the cell suspension every 10 min. The monocytes that had phagocytosed the carbonyl-iron were removed with a magnet.

- Adherence to plastic: PBMC (1 x 10^6 cells/ml) were incubated in RPMI-FCS in a 75 cm^2 incubation flask at 37 °C and 5% CO_2, for 45 min. This procedure was repeated one to three times with the non-adherent fraction of the PBMC.

- Dynabeads M450 (Dynal): Because of the presence of FcγR, monocytes are able to bind to beads coated with IgG. Immunomagnetic beads coated with sheep anti-mouse IgG (Dynabeads M450, Dynal, Oslo, Norway) were incubated with PBMC (2.5 x 10^6 cells/ml) at a bead concentration of 10^7 beads/ml at 37 °C for 60 min. with thoroughly resuspension of the cell suspension every 10 min. The rosettes of Dynabeads M450 and monocytes were eliminated by a magnet (according to the protocol provided by the manufacturer).

- Panning with anti-CD14 mAb: A petri dish was coated with affinity purified, Fc-specific, goat anti-mouse IgG (Cappel, Malvern, USA) at 4 °C for 18 h, and then incubated with 1% gelatin (as a blocking agent) in PBS at 37 °C for 2 hr. PBMC, 3 - 5 x 10^6 cells/ml were incubated at 4 °C for 1 hr with a mixture of anti-CD14 mAb (63D3, FMC17, AML 2-23 and WT14) or WT14 alone at saturating concentrations, washed twice and resuspended in RPMI-FCS. CD14-positive cells were then removed from the PBMC by capturing them on a dish coated with anti-mouse antibodies (1 hr at 37 °C).

In all cases, the cells remaining after the monocyte depletion procedure were tested in the mIgG2a anti-CD3 induced T cell proliferation assay. In several experiments, the percentage of contaminating monocytes was evaluated by FACS analysis. In some experiments, as described in the results and discussion section, a combination of depletion procedures was used.

T cell proliferation assay: The mIgG2a anti-CD3 induced T cell proliferation was measured by a standard [^{3}H]-thymidine incorporation assay (14) using either PBMC, or monocyte-depleted cell suspensions obtained from the procedures described above, or a mixture of purified T lymphocytes and graded numbers of purified monocytes. T lymphocytes were purified from PBMC by counterflow centrifugation (6) followed by rosetting with sheep erythrocytes that had been treated with aminoethyl-isothiouronium bromide (2). These purified T lymphocytes were cryopreserved and used later in the T cell proliferation assay. Purified monocytes were also obtained by counterflow centrifugation and used after cryopreservation. 10^5 PBMC or monocyte-depleted cells (or 10^5 purified T cells and various numbers of monocytes) were incubated in RPMI-FCS with anti-CD3 mAb (WT32, 50 ng/ml) at 37 °C in a humidified atmosphere of 5% CO_2 in air for 72 hr in U-bottomed microtitre wells. During the last 18 hr of the incubation period [^{3}H]-thymidine (1.85 x 10^4 Bq/well) was present. Experiments were carried out in triplicate. The stimulation-index (SI) was calculated by dividing counts per minute (c.p.m.) obtained in the presence of anti-CD3 mAb by c.p.m. obtained in its absence.

Immunofluorescence: 5 x 10^5 cells were incubated with mIgG2a mAb 197 or mIgG2b mAb anti-Leu-M3 (or no mAb for controls) at 4 °C for 30 min., washed in PBS containing 1% bovine serum albumin and 0.1% NaN_3, labelled with FITC-conjugated subclass-specific $F(ab')_2$ fragments of goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL), and measured in a Coulter Epics flow cytometer. In each sample the fluorescence intensity from 5000 cells was quantified.

RESULTS AND DISCUSSION

Sensitivity of the readout method

The T cell proliferation assay induced by mIgG2a anti-CD3 mAb is dependent on the presence of FcγRI-positive accessory cells. In previous experiments we had found that in the presence of 20% monocytes, a concentration of 50 ng/ml of WT32 (IgG2a anti-CD3 mAb) results in optimal T cell proliferation. We subsequently determined the percentage of contaminating monocytes that is able to induce a detectable T cell response in the presence of 50 ng/ml of IgG2a

anti-CD3 mAb (Figure 1). When 1000 monocytes were added to 10^5 purified T cells, there was already a measurable proliferative response to mIgG2a anti-CD3 mAb (605 ± 287 c.p.m.; SI = 15). Therefore, the presence of 1% contaminating monocytes can be detected using this assay. Optimal T cell proliferation was obtained when 5000 monocytes per 10^5 T cells were present.

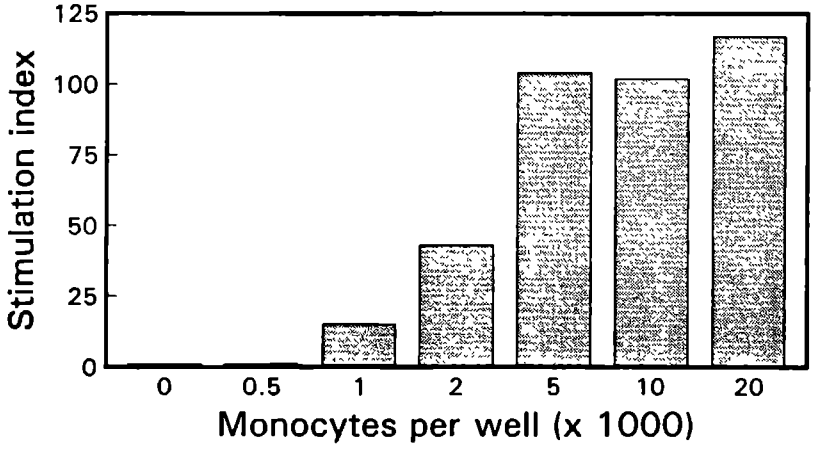

Figure 1.

Proliferation of purified T cells (1 x 10^5 cells/well) stimulated with mIgG2a anti-CD3 mAb (WT32, 50 ng/ml) in the presence of increasing numbers of autologous monocytes (0 - 2 x 10^4 cells). Results are expressed as stimulation indices. At an optimal concentration of monocytes (2 x 10^4), [^{3}H]-thymidine incorporation was 77,911 ± 4,881 c.p.m.

Monocyte depletion obtained with different procedures

Removal of monocytes by any of the procedures described above caused a significant reduction of monocytes as evidenced by a decreased mitogenic response to mIgG2a anti-CD3 mAb. T cell proliferation measured after monocyte depletion was compared with T cell proliferation obtained with the untreated suspension of PBMC, and is expressed as a percentage of the value obtained with untreated PBMC. The extent of monocyte depletion varied between different experiments, but was never complete with any of the depletion procedures when used on its own (Table 1).

Table 1.

Removal of monocytes from freshly isolated or cryopreserved PBMC by different procedures

	Proliferation of mononuclear cells			
	Freshly isolated		Cryopreserved	
Method of elimination	% of control	n[a]	% of control	n
Untreated	100	16	100	16
Counterflow centrifugation	31 ± 18	4	ND[b]	
Carbonyl-iron	32 ± 30	3	ND	
Adherence to plastic	36 ± 13	4	80 ± 5	3
Dynabeads M450	48 ± 12	2	ND	
Panning with mAb mixture	57 ± 18	4	29 ± 26	6
Panning with WT14	63 ± 20	3	36 ± 17	3

a) n: number of experiments
b) ND: not determined.

It is noteworthy that adherence to plastic, which is a common procedure for monocyte depletion, yielded unsatisfactory results, especially when applied to cryopreserved cells. FACS analysis showed that, with cryopreserved cells, the percentage of FcγRI-positive cells (stained with mAb 197) decreased from 23.3% to 11.2% after adherence, and the percentage of CD14-positive cells (stained with anti-Leu-M3) decreased from 19.9% to 8.3%. It has been demonstrated before that significant numbers of monocytes are non-adherent (7,11). Chehimi et al. (5) have compared several methods for the removal of monocytes, and concluded that combinations of adherence and phagocytosis of carbonyl-iron or adherence and panning over plates coated with immunoglobulin were insufficient to remove all monocytes. Only a combination of four depletion methods proved to be effective in their hands. Similarly, Ceuppens and Baroja (4) had to use a combination of E-rosetting, panning with an anti-monocyte mAb, and leucine methylester in order to obtain T cell suspensions that were completely devoid of accessory cells. With cryopreserved cells, we observed that panning with anti-CD14 mAb WT14 yielded better results than adherence,

although monocyte depletion was apparently still not complete (Table 1). Using FACS analysis, FcγRI-positive cells decreased from 26.4% to 2.6% (CD14-positive cells from 26.7% to 2.7%) after panning with WT14. The anti-CD14 mAb has been used before, with rabbit complement, as one of the steps in the removal of monocytes (5). The CD14 antigen appears to be important for monocyte function (12,16) and we have previously demonstrated that mAb WT14, which is directed against CD14, can be used in immunohistology as a diagnostic marker for macrophage infiltration during acute interstitial rejection of renal allografts (3). Although one might have expected that in a panning procedure a mixture of mAb, recognizing different epitopes on the CD14 antigen, would be more effective than using only one mAb, this appeared to be not the case since mAb WT14 was equally effective as a mixture of anti-CD14 mAb in our hands (Table 1). The failure to accomplish complete elimination of monocytes with the panning procedure alone using (a mixture of) anti-CD14 mAbs, may be related to heterogeneity of CD14 expression. It is of interest to note that a CD14-negative subset of monocytes has been described (9).

Monocyte depletion by a combination of two elimination procedures

None of the methods mentioned above was successful, when used separately, in obtaining complete elimination of the accessory monocytes that supported the mIgG2a anti-CD3 induced T cell proliferation. Since panning with anti-CD14 mAb reproducibly caused, by itself, a significant reduction in monocyte numbers (especially when cryopreserved mononuclear cells were used), we decided to test this method in combination with the phagocytosis of carbonyl-iron. First, PBMC were incubated with a mixture of anti-CD14 mAb or WT14 alone and most of the CD14-positive cells were removed by panning on a petri dish coated with (Fc-specific) goat anti-mouse IgG. The remaining cells (still containing 2-3% monocytes as mentioned above) were subsequently incubated with carbonyl-iron, and the phagocytic cells were removed by a magnet. The results are shown in Figure 2. When a combination of panning with a mixture of anti-CD14 mAbs (or WT14 alone) and phagocytosis of carbonyl-iron was applied to freshly isolated PBMC, a reproducible reduction of 97 $\pm$ 2% (n=4) and 98 $\pm$ 1% (n=2), respectively, of the mIgG2a anti-CD3 induced T cell proliferation was achieved. Therefore, panning with a mixture of anti-CD14 mAbs (or WT14 alone) followed by phagocytosis of carbonyl-iron consistently resulted in a virtually complete elimination of monocytes. The observed reduction of T cell proliferation could not be ascribed to some nonspecific toxic

effect of the depletion procedure, since the remaining cell suspension was still able to proliferate in response to mIgG2a anti-CD3 mAb when purified monocytes were added (Figure 2).

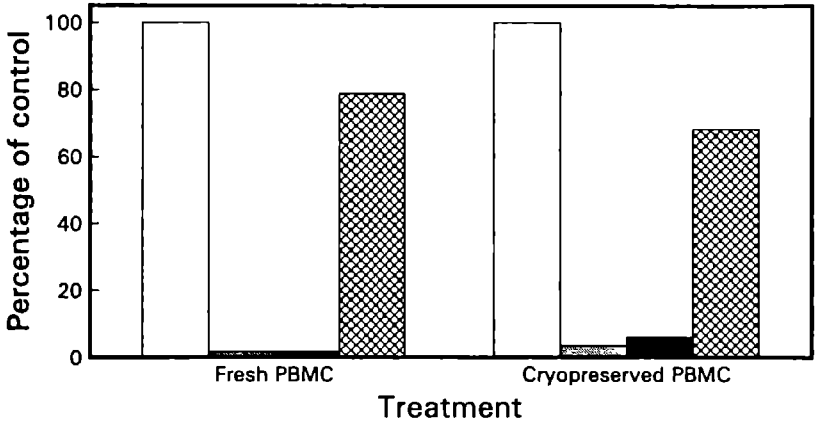

Figure 2.

Elimination of monocytes and macrophages from freshly isolated or cryopreserved PBMC. Results are expressed as a percentage of the T cell proliferation obtained with untreated cells (open bars). Monocytes were depleted by the combination of panning with a mixture of anti-CD14 mAb and phagocytosis of carbonyl-iron (shaded bars), or by a combination of panning with WT14 alone and phagocytosis of carbonyl-iron (black bars). In some experiments, monocytes were added again (to a final concentration of 20%), after depletion by panning with WT14 plus carbonyl-iron (cross-hatched bar). The figure contains the combined data from a total of 16 experiments. T cell proliferation obtained with untreated PBMC ranged from 13,591 to 57,834 c.p.m.

It is important to stress that the method described here is also effective in depleting monocytes from cryopreserved cell suspensions (Figure 2). This conclusion was supported by immunofluorescence studies. After panning with WT14 and phagocytosis of carbonyl-iron, the percentage of FcγRI-positive cells had decreased from 26.4% to 1.0% (CD14-positive cells from 26.7% to 0.8%). In our studies of the FcγR for mIgG2b, we found that only a small percentage ("mIgG2b high-responders") of normal individuals have a functional receptor for this isotype of mouse immunoglobulins, and therefore it is difficult to obtain

freshly isolated PBMC. Cryopreservation of cells is very helpful in such circumstances. When the monocyte depletion method described above was applied to cryopreserved PBMC from "mIgG2b high-responders", a significant mitogenic response to mIgG2b anti-CD3 mAb was still observed which indicates that cells other than monocytes (presumably B lymphocytes) can function as accessory cells in this model (10).

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A polymorphic Fc receptor for mouse IgG2b on human B cells and monocytes

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ABSTRACT

With respect to murine (m)IgG1 anti-CD3 monoclonal antibody (mAb), a polymorphic mitogenic response of peripheral blood mononuclear cells (PBMC) has been described which is caused by polymorphism of monocyte FcγRII, and which defines high-responders to mIgG1 (mIgG1-HR, approximately 70% of normal individuals) and low-responders (mIgG1-LR). PBMC also exhibit a polymorphic mitogenic response to mIgG2b anti-CD3 mAb. In the present study 18 out of 550 individuals (3%) were mIgG2b-HR. Purified monocytes from mIgG2b-HR were able to support the mitogenic response to mIgG2b anti-CD3 mAb of purified T cells from mIgG2b-LR. Surprisingly, a significant mitogenic response to mIgG2b anti-CD3 mAb remained after vigorous depletion of monocytes from mIgG2b-HR PBMC. Apparently B cells are responsible for this accessory function since Epstein-Barr virus (EBV)-transformed B cells from mIgG2b-HR (but not from mIgG2b-LR) were able to support T cell proliferation induced by mIgG2b anti-CD3 mAb. Only EBV-B cells from mIgG2b-HR were able to form rosettes with human red blood cells (RBC) that had been sensitized with mIgG2b anti-glycophorin A mAb (EA-mIgG2b). These EBV-B cells did not express FcγRI or FcγRIII, and could bind some but not all anti-FcγRII mAb. The mitogenic response to mIgG2b anti-CD3 was not inhibited by any of the anti-FcγRII mAb. From these studies we conclude that a polymorphic Fc receptor is expressed on human B cells and monocytes, which cross-reacts with mIgG2b. This receptor is different from FcγRI and FcγRIII, and apparently also from FcγRII.

INTRODUCTION

Fc receptors for IgG (FcγR) are involved in clearing immune complexes, phagocytosis, antibody dependent cell-mediated cytotoxicity (ADCC), and can act as a trigger molecule for the extracellular release of lysosomal enzymes and superoxide production. Until now three classes of human FcγR have been described, FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). The availability of mAb, and the isolation of cDNA coding for these Fc receptors, have rapidly expanded our knowledge of structure and function of human Fc receptors (1-5). FcγRI, a 72 kDa glycoprotein with high-affinity for monomeric human (h) IgG, is present on monocytes and macrophages from virtually all individuals and can be induced on neutrophils after stimulation with interferon-γ (IFNγ) (6). FcγRII, a 40 kDa glycoprotein with low-affinity for hIgG is present on monocytes, macrophages, neutrophils, eosinophils, basophils, platelets and B cells. This widely distributed receptor occurs in multiple isoforms (7-9). Proteolytic enzymes can significantly enhance the affinity of FcγRII (10,11). Another receptor class, FcγRIII (50 - 80 kDa glycoprotein) occurs in two forms: a transmembrane form which is found on macrophages and natural killer (NK) cells, and a phosphatidylinositolglycan-linked receptor which is abundantly expressed on neutrophils (12).

Human FcγR can also cross-react with mouse IgG. This cross-reactivity may become important when mouse monoclonal antibodies (mAb) are used for immunotherapy. Major differences between different isotypes of murine IgG have been observed with respect to their efficacy to mediate ADCC by human monocytes (13) or lymphocytes (14). mIgG2a can bind to FcγRI, whereas mIgG1 can bind to FcγRII (15). With respect to the binding of mIgG1 to FcγRII, a polymorphism has been described. This mIgG1 polymorphism was first observed using mIgG1 anti-CD3 mAb in T cell proliferation assays, where FcγR-positive cells function as accessory cells. In 70% of Caucasian individuals, monocyte FcγRII exhibits strong binding of mIgG1 (mIgG1 high-responder, HR) and in 30% of the population this interaction is weak (mIgG1 low-responder, LR) (4,11,16-18). This polymorphism is also reflected in EA-mIgG1 rosetting (17) and in monocyte ADCC against erythrocytes that have been coated with mIgG1 antibody (19,20). Three phenotypical isoelectric focusing patterns have been found with monocyte FcγRII, indicating homozygous forms of mIgG1-HR, mIgG1-LR and a heterozygous form (21).

Allotypic isoforms of this receptor can also be detected using a new anti-FcγRII mAb, 41H.16 (22). DNA sequence analysis predicts differences in only one or two amino acid residues between mIgG1-HR and mIgG1-LR receptor molecules (23,24).

We and others have previously reported that only a small minority (less than 10%) of normal human individuals are high-responders with respect to mIgG2b (mIgG2b-HR) in the anti-CD3 induced T cell proliferation assay (18,25-28). Remarkably, the T cell proliferation induced by mIgG2b anti-CD3 in mIgG2b-HR is not associated with release of IFNγ or IL2, in contrast to T cell activation induced by mIgG2a or mIgG1 anti-CD3 mAb (29). This striking difference suggests the existence of a completely different T cell activation pathway associated with binding of the mIgG2b antibody to an Fc receptor different from FcγRI or FcγRII. In this study we have therefore tried to characterize the FcγR which is reactive with mIgG2b. The results suggest that the polymorphic Fc receptor for mIgG2b is present on B cells as well as monocytes, and that the binding site for mIgG2b is different from the binding site for mIgG1, and might even be located on a different receptor.

MATERIALS AND METHODS

Monoclonal antibodies: Anti-FcγRI mAb 32.2 (mIgG1) and 197 (mIgG2a), and anti-FcγRII mAb IV.3 (mIgG2b) were obtained from Medarex (West Lebanon, NH). Anti-FcγRII mAb CIKM5 (mIgG1) (30) was a generous gift from dr. G. Pilkington (Peter MacCullem Cancer Institute, Melbourne, Australia); anti-FcγRIII mAb, Leu 11a (mIgG1) and Leu 11b (IgM) were from Becton Dickinson (Mountain View, CA). The mAb KB61 (mIgG1) (31) and 41H.16 (mIgG2a) (32) were kindly provided by Drs. D.Y. Mason (John Radcliffe Hospital, Oxford, U.K.) and Th.F. Zipf (Anderson Hospital and Tumor Institute, Houston, TX), respectively. These last two mAb were included in the CD32 cluster during the Fourth Leucocyte Workshop (Vienna, 1989) but, in contrast to IV.3 and CIKM5, these two mAb recognize an isoform of the CD32 antigen that is expressed preferentially on B lymphoid cells (31,32). Anti-CD3 mAb SPV-T3a (33) was a kind gift from Dr. H. Spits (DNAX, Palo Alto, CA). Anti-CD3 mAb WT32 (16) and anti-T cell receptor (TcR) mAb WT31 (16,34) were developed in our own laboratory. The following anti-CD14 mAb were used: 63D3 (cell line obtained from American Type Culture Collection, Rockville, MD), FMC17 (Seralab, Sussex, U.K.), AML-2-23 (Medarex) and WT14 (from our own laboratory) (35). A mIgG2b anti-glycophorin A mAb was generously provided by Dr. L. Aarden (CLB, Amsterdam, The Netherlands).

Monocytes: Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats or from leucocytes isolated by cytapheresis from healthy donors, by centrifugation on a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden). Monocytes were isolated from PBMC by counterflow centrifugation (36) and were >90% pure, as judged by staining with May-Grünwald-Giemsa and for non-specific esterase. PBMC and purified monocytes were cryopreserved and used later. The recovery and viability of the cryopreserved cells was >70% and >95%, respectively.

T lymphocytes: Lymphocytes were purified from PBMC by counterflow centrifugation. T lymphocytes were isolated from this suspension by rosetting with sheep erythrocytes treated with aminoethylisothiouronium bromide (37). The purified T lymphocytes were cryopreserved and used later.

EBV-transformed B cell lines: Cryopreserved PBMC from some mIgG2b-HR or -LR individuals were used for Epstein-Barr virus (EBV) transformation of the B cells. Immortalization and stabilization of the cell lines were performed by dr. F. Uytdehaag and co-workers at the Department for Public Health (RIVM) at Bilthoven, The Netherlands. EBV-transformed B cell lines, human erythromyeloid (K562), myeloid (U937) and B-lymphoblastoid (Daudi, Raji) cell lines were cultured in RPMI-1640 medium containing Hepes and sodium bicarbonate, supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate and gentamycin (40 μg/ml). Since infection of the cells with *Mycoplasma* species can cause false-positive FcR reactivity (38), the cell lines K562, U937, Daudi, Raji, and all the EBV-transformed B cell lines, were tested for *Mycoplasma* infection using the Gen-Probe Mycoplasma Tissue Culture II detection system (Gen-Probe, San Diego, CA) which contains a universal DNA-probe that can hybridize all known *Mycoplasma* species. Cell lines that were *Mycoplasma*-positive were first treated with ciprofloxacin during 14 days (39), and only *Mycoplasma*-negative cells were used in the present study. PBMC, monocytes and EBV-transformed B cell lines were given the same identification number as the individual from which they were derived.

Depletion of monocytes: Depletion of monocytes from PBMC was carried out by using a combination of panning with anti-CD14 mAb and phagocytosis of carbonyl-iron. A Petri dish was coated with affinity-purified, Fc specific goat-anti-mouse IgG (Cappel, Malvern, PA) at 4°C for 18 hr and incubated with 1% gelatin at 37°C for 2 hr. PBMC (2.5 x 10^6 cells/ml) were incubated at 4°C for 1 hr with a mixture of anti-CD14 mAb (mAb 63D3, FMC17, AML-2-23, and WT14) at saturating concentrations, washed twice and resuspended in RPMI-1640 containing 10% FCS. After incubating for 1 hr at 37°C in the precoated Petri dish, non-adherent cells were removed and incubated (at 5 - 10 x 10^6 cells/ml) with 40 mg/ml carbonyl-iron powder for 1 hr at 37°C with thoroughly resuspending the cell suspension every 10 min. The monocytes which had phagocytosed the carbonyl-iron were removed magnetically. The monocyte-depleted cell suspension did not contain detectable numbers of

monocytes as measured in immunofluorescence (using mAb WT14), or seen on a cytospin-preparation using May-Grünwald-Giemsa staining.

T cell proliferation assay: T cell proliferation was measured by a standard [^{3}H]-thymidine incorporation assay (16) using either PBMC, or monocytes plus purified autologous T cells, or EBV-transformed B cells plus purified autologous T cells. Cells were incubated with mouse anti-CD3 or anti-TcR mAb of different subclasses. Briefly, 1 x 10^5 PBMC or 1 x 10^5 purified T cells plus 2 x 10^4 accessory cells (autologous monocytes or EBV-transformed B cells) were incubated with anti-CD3 or anti-TcR mAb for 72 hr at 37°C and 5% CO_2 in U-bottomed microtitre wells. During the last 18 hr of the incubation period [^{3}H]-thymidine (1.85 x 10^4 Bq/well) was present. Anti-CD3/TcR mAb used were WT32 (mIgG2a) at 50 ng/ml, SPV-T3a (mIgG2b) at 1 μg/ml, or WT31 (mIgG1) at 100 ng/ml. The stimulation-index (SI) was calculated by dividing counts per minute (c.p.m.) in the presence and absence of anti-CD3 mAb. High-responsiveness was defined by a SI > 5. In some experiments anti-FcγRII mAb (10 μg/ml) were added at the start of the T cell proliferation assay. When EBV-transformed B cells, or cell lines Daudi, K562, or U937 were used as accessory cells in T cell proliferation assays, these cells were first irradiated at 60 Gy. All proliferation experiments were performed in triplicate at least five times.

EA-rosetting: Rh(D)-positive human red blood cells (RBC) were sensitized with anti-glycophorin A mAb of the mIgG2b isotype. EBV-B cells (2 x 10^6 cells/ml) were incubated for 1 hr at room temperature with 0.5% (vol/vol) sensitized human RBC after centrifugation for 4 min. at 20°C (20 x *g*). The percentage of rosettes (at least three bound human RBC) was scored microscopically. Rosetting at low ionic strength (40) was carried out in a medium consisting of a mixture of one part of phosphate-buffered saline (PBS) and two parts of a 5% glucose suspension (pH 7.4). In some experiments we used Rh(D)-positive human RBC sensitized with either mIgG1 anti-glycophorin A mAb (EA-mIgG1) (a gift from M. Bos, CLB, Amsterdam, The Netherlands) or human IgG, a commercially available polyclonal anti-Rh(D) antiserum containing mainly hIgG3 and hIgG1 (Merz & Dade, Düdingen, Switzerland) to detect FcγRII or FcγRI, respectively (15). The presented experiments were performed at least five times.

Immunofluorescence assay and flow cytometry: Immunofluorescence studies were carried out using standard procedures. Briefly, 5 x 10^5 cells were incubated with mAb on ice for 30 min., washed in PBS containing 1% BSA and 0.1% NaN_3, labelled with FITC-conjugated $F(ab')_2$ fragments of sheep anti-mouse IgG H&L chain (Cappel, Malvern, PA) and measured in an Ortho 50-H flow cytometer. In each sample the fluorescence intensity from 5000 cells was quantitated.

RESULTS

Only few individuals are mIgG2b-HR

In a search for mIgG2b-HR individuals, we performed T cell proliferation assays in 550 different mononuclear cell suspensions. A proliferative response to mIgG2b anti-CD3 mAb was observed with PBMC from 18 (3%) of the tested individuals. All the individuals tested responded to mIgG2a anti-CD3, which indicates the presence of FcγRI. Table 1 shows the results of the proliferation assays of four mIgG2b-HR individuals whom we have studied in detail. These four individuals were also high-responder to mIgG1.

Table 1.

Anti-CD3 induced T cell proliferation in PBMC from four mIgG2b-HR individuals (I-IV)

	Stimulation index			
Subclass anti-CD3 mAb	I	II	III	IV
No mAb	1	1	1	1
mIgG1	22	26	18	22
mIgG2a	25	56	48	29
mIgG2b	19	35	48	32

PBMC (1 x 10^5 cells) were incubated with anti-CD3/TcR mAb of different subclass: WT32 (mIgG2a), 50 ng/ml; SPV-T3a (mIgG2b), 1 μg/ml or WT31 (mIgG1), 100 ng/ml. In the T cell proliferation assay [^{3}H]-thymidine incorporation was measured during the last 18 hr of the incubation period of 72 hr. The stimulation-index was calculated by dividing c.p.m. obtained in the presence and absence of anti-CD3/TcR mAb. The experiments were performed in triplicate at least five times.

Monocytes can support T cell proliferation induced by mIgG2b anti-CD3 mAb

We next tested whether high-responsiveness to mIgG2b is a property of the T cells, or is determined by the accessory cells. Table 2 shows the results of an experiment in which monocytes (obtained by elutriation) from a mIgG2b-HR or mIgG2b-LR individual were incubated with (non-autologous) T cells from a mIgG2b-HR or -LR in the presence of mIgG2b anti-CD3 mAb. No T cell proliferation was seen in the absence of accessory cells, and only monocytes from mIgG2b-HR could support the mitogenic response.

Table 2.

mIgG2b anti-CD3 induced T cell proliferation of purified T cells from mIgG2b-HR or mIgG2b-LR in the presence of (non-autologous) mIgG2b-HR or mIgG2b-LR monocytes

T cells	Monocytes	[^{3}H]-Thymidine incorporation (c.p.m.)	
		No anti-CD3	+ anti-CD3
LR	LR	54 ± 7	88 ± 23
HR	LR	2,518 ± 682	785 ± 391
LR	HR	99 ± 35	6,349 ± 655
HR	HR	1,911 ± 413	13,075 ± 250

Purified T cells (1 x 10^5 cells) plus purified (non-autologous) monocytes (2 x 10^4 cells) from mIgG2b-HR or mIgG2b-LR individuals were incubated with mIgG2b anti-CD3 mAb (SPV-T3a, 1 μg/ml). The [^{3}H]-thymidine incorporation was measured during the last 18 hr of the incubation period of 72 hr. T cells or monocytes incubated seperately with mIgG2b anti-CD3 gave no response (< 100 c.p.m.). Values given are means ± SD of triplicates.

In order to further characterize the polymorphic Fc receptor for mIgG2b that apparently is present on human monocytes, the anti-FcγRII mAb IV.3, KB61 and 41H.16 were tested for inhibition of the mIgG2b anti-CD3 induced mitogenic response. Whereas mIgG1 anti-CD3 induced T cell proliferation

could be inhibited almost completely by these antibodies, the mitogenic response to mIgG2b anti-CD3 mAb was not inhibited at all (Table 3). Inhibition experiments were also performed at lower concentrations of mIgG2b anti-CD3 (0.1 and 0.01 μg/ml). Also under these conditions no significant inhibition by anti-FcγRII mAb was observed. At 0.01 μg/ml of mIgG2b anti-CD3, for instance, the mean percentage of inhibition observed in three experiments was 27% for mAb IV.3, 19% for KB61, and 20% for 41H.16. Even the combination of mAb IV.3 and 41H.16 was not inhibitory for the mIgG2b-induced response (the percentages of inhibition obtained in three experiments were 1%, 5%, and 32%). When instead of PBMC mIgG2b-HR monocytes (separated from PBMC by elutriation) plus purified autologous T cells were used, an identical pattern of inhibition by anti-FcγRII mAb was observed (complete inhibition of mIgG1 response, no inhibition of mIgG2b response; data not shown).

Table 3.

Inhibition by anti-FcγRII mAb of the mitogenic response to mIgG1 or mIgG2b anti-CD3 mAb from PBMC of two mIgG2b-HR individuals

		Inhibition (%) of T cell proliferation	
	Anti-FcγRII mAb	mIgG1	mIgG2b
Donor I	IV.3	95	0
	KB61	66	4
	41H.16	97	18
Donor II	IV.3	91	4
	KB61	75	11
	41H.16	97	16

PBMC (1 x 10^5 cells) from two mIgG2b-HR individuals were incubated with 10 μg/ml anti-FcγRII mAb (IV.3, KB61 or 41H.16) during 30 min. before mIgG1 anti-CD3 mAb (WT31, 100 ng/ml) or mIgG2b anti-CD3 mAb (SPV-T3a, 1 μg/ml) were added. The [^{3}H]-thymidine incorporation was measured during the last 18 hr of the incubation period of 72 hr. The percentage inhibition of T cell proliferation was calculated.

Other cells beside monocytes can function as accessory cells for mIgG2b response

In order to test whether monocytes were the only cell type capable of supporting mIgG2b-induced T cell proliferation, monocytes were depleted from the PBMC of a mIgG2b-HR by a combination of panning with anti-CD14 mAb and phagocytosis of carbonyl-iron. This procedure resulted in a complete elimination of the mIgG2a anti-CD3 induced T cell response (Table 4). T cells were still viable as evidenced by the fact that the response to mIgG2a anti-CD3 could be completely restored (32,396 ± 1,487 c.p.m.) by addition of monocytes. The mitogenic response to mIgG2b anti-CD3 mAb was not affected by monocyte depletion in the experiment demonstrated in Table 4, although in three other experiments the response to mIgG2b was decreased to some extent: 50 - 90% of the mIgG2b response observed with PBMC was still present after complete removal of monocytes.

Table 4.

Anti-CD3 induced T cell proliferation of PBMC or monocyte-depleted PBMC from a mIgG2b-HR individual.

	Proliferative response (c.p.m.)	
Anti-CD3 mAb subclass	PBMC	Monocyte-depleted
No mAb	1,253 ± 343	242 ± 34
mIgG1	13,617 ± 1,813	1,140 ± 222
mIgG2a	33,109 ± 2,102	290 ± 69
mIgG2b	8,693 ± 1,153	7,625 ± 1,569

PBMC or monocyte-depleted PBMC (1 x 10^5 cells) from a mIgG2b-HR were incubated with anti-CD3/TcR mAb of different subclass; WT32, mIgG2a (50 ng/ml); SPV-T3a, mIgG2b (1 μg/ml); or WT31, mIgG1 (100 ng/ml). In the T cell proliferation assay [^{3}H]-thymidine incorporation (c.p.m.) was measured during the last 18 hr of the incubation period of 72 hr. Monocytes were depleted from PBMC using panning with anti-CD14 mAb and carbonyl-iron, as described in Materials and Methods. The results are given as means ± SD of triplicate determinations.

Therefore, not only monocytes can support the mitogenic response to mIgG2b anti-CD3 mAb but also another cell type that is still present in the monocyte-depleted cell suspension.

Accessory cell function of EBV-transformed B cells

In preliminary experiments we had observed that B cell lines Daudi and Raji could support the mitogenic response to mIgG2b anti-CD3 mAb. In contrast, no accessory cell function was measured with the (erythro)-myeloid lines K562, U937 and HL-60 (data not shown). Therefore, B cells may be responsible for the accessory cell function and support the mIgG2b anti-CD3 induced T cell proliferation observed after monocytes depletion. We tested the accessory cell function of EBV-transformed B cells from mIgG2b-HR and -LR individuals (Figure 1). A proliferative response of T cells to mIgG2b anti-CD3 mAb was found when autologous EBV-B cells from mIgG2b-HR were used as accessory cells, but not when EBV-B cells from mIgG2b-LR were used.

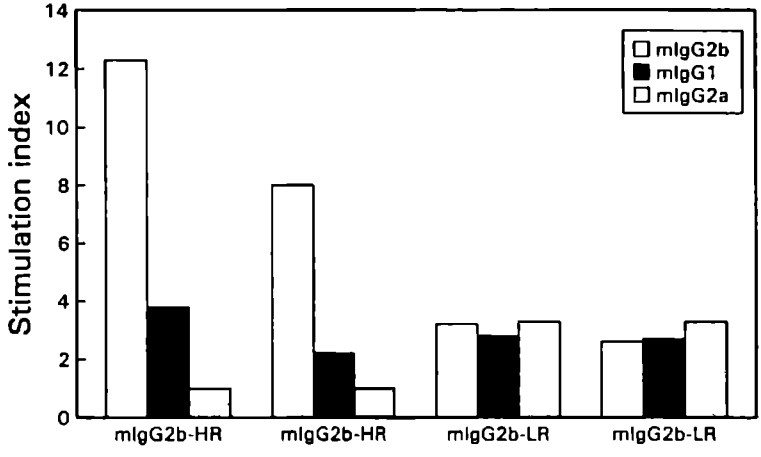

Figure 1.

Mitogenic response of T cells (1 x 10^5) to anti-CD3 mAb of different subclass (WT32, mIgG2a; SPV-T3a, mIgG2b; or WT31, mIgG1) in the presence of autologous EBV-B cells (2 x 10^4) originating from mIgG2b-HR or mIgG2b-LR individuals. Stimulation indices were calculated by dividing c.p.m. obtained in the presence and absence of anti-CD3/TcR mAb.

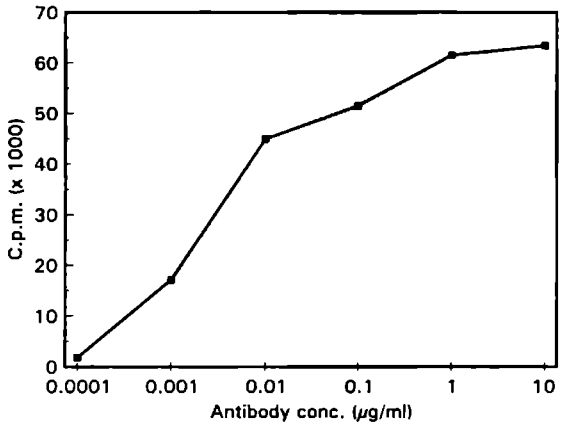

Figure 2.

Dose-response curve of T cell proliferation (1 x 10^5 T cells) induced by increasing concentrations of mIgG2b anti-CD3 mAb (SPV-T3a; 0.1 ng/ml - 10 μg/ml) in the presence of autologous EBV-transformed B cells (2 x 10^4 cells) from mIgG2b-HR. [^{3}H]-thymidine incorporation was measured during the last 18 hr of the incubation period of 72 hr. Values given are means of triplicates.

Table 5.

Anti-FcγRII mAb does not inhibit mIgG2b anti-CD3 induced T cell proliferation supported by autologous EBV-B cells from mIgG2b-HR

	Inhibition (%) of T cell proliferation		
Anti-FcγRII mAb	Donor I	Donor II	Donor III
IV.3	5	0	14
KB61	10	0	10
41H.16	20	9	10

EBV-B cells (2 x 10^4 cells) from three different mIgG2b-HR individuals were incubated with 10 μg/ml anti-FcγRII mAb (IV.3, KB61 or 41H.16) during 30 min. before purified autologous T cells (1 x 10^5 cells) and mIgG2b anti-CD3 mAb (SPV-T3a, 1 μg/ml) were added. [^{3}H]-thymidine incorporation was measured during the last 18 hr of the incubation period of 72 hr. The percentage inhibition of T cell proliferation was calculated.

Significant proliferation of T cells in the presence of mIgG2b-HR EBV-B cells was already observed at 1 ng/ml of mIgG2b anti-CD3 (Figure 2). EBV-transformed B cells (from either mIgG2b-HR or mIgG2b-LR) did not support the T cell response to mIgG1 or mIgG2a anti-CD3 mAb (Figure 1). Furthermore, the mitogenic response to mIgG2b anti-CD3 supported by the EBV-B cells from mIgG2b-HR was not inhibited by anti-FcγRII mAb KB61 and 41H.16 (Table 5). Besides, in immunofluorescence studies, EBV-B cells from mIgG2b-HR and mIgG2b-LR showed a positive staining with KB61 and 41H.16 but they were negative with the anti-FcγRII mAb IV.3 and CIKM5. In addition, no staining was seen when anti-FcγRI mAb (32.2 and 197.1) or anti-FcγRIII mAb (Leu 11a and Leu 11b) were used (data not shown).

Table 6.

EA-mIgG2b rosetting by EBV-B cells originating from mIgG2b-HR and mIgG2b-LR

	Percentage rosetting cells	
	At normal ionic strength	At low ionic strength
EBV-HR I	10 ± 3	33 ± 2
EBV-HR II	16 ± 5	43 ± 5
EBV-HR III	44 ± 4	77 ± 2
EBV-HR IV	40 ± 5	69 ± 1
EBV-LR 1	0	0
EBV-LR 2	0	0
EBV-LR 3	0	0

EBV-B cells (2 x 10^6 cells/ml) from mIgG2b-HR and mIgG2b-LR individuals were incubated with 0.5% (vol/vol) HRBC sensitized with anti-glycophorin A mAb of the mIgG2b isotype. The EBV-B cells were incubated at room temperature at normal or at low ionic strength (see the Materials and Methods). The percentage of rosettes was scored microscopically. The means ± SD of five individual experiments are presented.

EA-mIgG2b rosetting of EBV-B cells from mIgG2b-HR individuals

Our assumption that a polymorphic Fc receptor for mIgG2b is present on human EBV-B cells was further supported by rosetting experiments. Human RBC coated with mIgG2b anti-glycophorin A mAb (EA-mIgG2b) formed rosettes with the EBV-B cells from mIgG2b-HR but not from mIgG2b-LR (Table 6). The percentage of rosettes increased when rosetting was performed in low ionic strength medium, whereas EBV-B cells from mIgG2b-LR remained negative. No rosettes were seen with EA-mIgG1 or EA-hIgG, detecting FcγRII and FcγRI, respectively, or with non-sensitized human RBC (data not shown).

DISCUSSION

Polymorphism of the mitogenic response to mIgG2b anti-CD3 mAb has been observed in several studies. Although no mIgG2b-HR were found in two groups of 20 and 12 individuals, respectively (33,41), we have previously reported that two individuals out of a group of 30 were mIgG2b-HR (18), and a similar frequency (3/27 and 3/30, respectively) was found in two other studies (25,27). In the present study we found 18 mIgG2b-HR among 550 individuals (3%). With respect to mIgG1, the percentage of high-responders is much higher, approximately 70% (16,25,27). Two mIgG2b-HR individuals have been reported to be mIgG1-LR (18,25), indicating that high-responsiveness with respect to mIgG2b is independent of the mIgG1-responder status. Furthermore, we have obtained evidence in recent studies that the mechanism of mIgG2b anti-CD3 induced T cell proliferation is different from mIgG1 or mIgG2a induced proliferation, since with mIgG2b anti-CD3 no production of IL2 or IFNγ was observed, and T cell proliferation was not inhibited by anti-IL2R mAb (29).

We first investigated whether the high-responsiveness to mIgG2b is determined by accessory cells, as is the case for mIgG1 high-responsiveness. Purified T cells from mIgG2b-HR individuals could not support the mitogenic response to mIgG2b anti-CD3. In the presence of mIgG2b-HR monocytes, however, purified T cells from a mIgG2b-LR could be induced to proliferate by mIgG2b anti-CD3. Therefore, monocytes can function as accessory cells for the mIgG2b anti-CD3 induced T cell proliferation.

Depletion of monocytes from PBMC results in a complete elimination of T cell

proliferation induced by mIgG2a or mIgG1 anti-CD3 mAb. By contrast, a significant response to mIgG2b was still observed after complete depletion of monocytes from mIgG2b-HR PBMC. This indicates that, apart from monocytes, other cells present in the mononuclear cell suspension have the capacity to support mIgG2b anti-CD3 induced T cell proliferation. Because B lymphocytes express Fc receptors, and since in preliminary experiments B cell lines Daudi and Raji could support the T cell response to mIgG2b, we tested the accessory function of EBV-transformed B cells derived from mIgG2b-HR or mIgG2b-LR individuals. Indeed, EBV-B cells from mIgG2b-HR (but not from mIgG2b-LR) could function as accessory cells for the mIgG2b response. To our knowledge, human B cells have not been tested before in this model. Mouse B cell blasts (obtained after stimulation with lipopolysaccharide, LPS) have been reported to be excellent accessory cells which support mIgG2b anti-CD3 induced proliferation of human T cells, although resting mouse B cells were ineffective (28).

The availability of EBV-transformed cells allowed us to investigate whether the accessory function is related to the presence of an Fc receptor for mIgG2b. We developed a rosetting technique employing a mIgG2b mAb directed against glycophorin A, which is abundantly expressed on human erythrocytes. The sensitizing antibody that we used is an isotype switch-variant of the mIgG1 mAb that we have previously used in our studies on mIgG1 polymorphism (17). Consistently, we observed rosetting (at a low percentage) with mIgG2b-HR EBV-B cells whereas no rosetting was observed with mIgG2b-LR EBV-B cells. Jones et al. (40) have reported that the binding of heat-aggregated mIgG2b to cell lines U937 and Daudi was strongly enhanced at low ionic strength. We therefore tested the effect of ionic strength, and found that the percentage of EA-mIgG2b rosettes obtained with EBV-transformed B cells from mIgG2b-HR could also be enhanced significantly when medium of low ionic strength was used, whereas mIgG2b-LR EBV-B cells remained negative. These findings demonstrate that an Fc receptor for mIgG2b is present on human EBV-transformed B cells, and that at normal ionic strength, this receptor apparently has a low-affinity for mIgG2b.

In immunofluorescence studies, we found no evidence for the presence of FcγRI or FcγRIII on EBV-B cells from mIgG2b-HR or mIgG2b-LR. Furthermore, mAb IV.3 and CIKM5 (which recognize monocyte FcγRII) did not bind either. On the other hand, mAb KB61 and 41H.16 (which also bind to an isoform of FcγRII that is expressed on B cells (31) did bind to EBV-B

cells from both mIgG2b-HR and mIgG2b-LR individuals. However, when mAb IV.3, KB61, and 41H.16 were tested for inhibition of anti-CD3 induced T cell proliferation (using mIgG2b-HR PBMC), the response to mIgG1 was strongly or even completely inhibited whereas the mIgG2b response was not affected. Similar results were obtained when purified monocytes or EBV-B cells from mIgG2b-HR individuals were used as accessory cells. Since the epitope recognized by IV.3 is different from the epitope(s) recognized by KB61 and 41H.16 (31), and none of these mAb caused any inhibition of the mIgG2b anti-CD3 induced T cell proliferation, it seems unlikely that FcγRII is involved in the mIgG2b polymorphism described here. Nevertheless, the possibility cannot be completely ruled out that mIgG2b might bind to a binding site on FcγRII that is not affected by binding of any of the anti-FcγRII mAb tested in this study.

It is often assumed that FcγRII is the Fc receptor responsible for the binding of (polymeric) mIgG2b to human cells. This assumption is partly based on the specificity of the murine macrophage FcγRII which binds both mIgG1 and mIgG2b (42). Furthermore, binding of aggregated mIgG2b to the human myeloid cell line U937 is inhibited by anti-FcγRII mAb IV.3 (40). Since IV.3 is a mIgG2b mAb, however, this inhibition may be due to the fact that, after binding to FcγRII, the Fc moiety of IV.3 binds to an Fc receptor for mIgG2b, as pointed out already by the authors (40). Furthermore, cell line U937 may not in all aspects be a valid model for human monocytes (and certainly not for human B cells). In this respect, it should be stressed that, despite the reported binding of aggregated mIgG2b (40), we observed that U937 could not support mIgG2b anti-CD3 induced T cell proliferation whereas it could support mIgG1 or mIgG2a anti-CD3 induced proliferation. Another remarkable finding bearing on this discussion, comes from studies on monocyte cytotoxicity (ADCC). The mIgG2b polymorphism observed in T cell proliferation assays was not reflected in ADCC: mIgG2b-HR and mIgG2b-LR monocytes were equally effective in cytotoxicity against mIgG2b-sensitized erythrocytes (20). These findings could be interpreted as evidence for the presence on human monocytes of two binding sites for mIgG2b: one binding site (involved in supporting T cell proliferation) that is polymorphic with respect to binding of mIgG2b, and another one (FcγRII?) that is not polymorphic in its binding of mIgG2b. It is noteworthy that monocyte cytotoxicity against mIgG2b-sensitized erythrocytes was only partially inhibited when both FcγRI and FcγRII had been blocked by mAb (20), suggesting that another Fc receptor with affinity for mIgG2b is present on human monocytes.

In the mouse, multiple isoforms of FcγRII that differ in their functional capacities occur on macrophages and B cells (43,44). Multiple FcγRII isoforms have also been described in humans (7,8,31). At this moment, it is not clear whether the polymorphic binding site for mIgG2b is present on one or more of these FcγRII isoforms or on a different Fc receptor. The lack of inhibition by anti-FcγRII mAb would argue against the first explanation. The functional difference that we have observed between anti-CD3 mAb of mIgG1 and mIgG2b isotype with regard to the dependence of T cell proliferation on IL2 (29) also suggests that the receptor structure binding mIgG2b may be different from FcγRII. Further analysis of the mIgG2b polymorphism at the DNA level will be necessary to answer these questions. The availability of EBV-transformed B cell lines expressing this polymorphic receptor will certainly facilitate these investigations.

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The human Fc receptor for mouse IgG2b on monocytes and EBV-B cells is functionally inhibited by anti-HLA class II antibodies

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ABSTRACT

We have recently described a polymorphic Fc receptor for murine IgG2b (mIgG2b), present on human monocytes and EBV-transformed B lymphocytes. The present study shows that anti-HLA class II monoclonal antibody (mAb) completely inhibits both the (Fc receptor-dependent) T cell proliferation, induced by mIgG2b anti-CD3 mAb, and rosetting with mIgG2b-sensitized erythrocytes. This inhibition is also observed with $F(ab')_2$ fragments of anti-HLA class II mAb, and is therefore not Fc-mediated. The Fc receptor for mIgG2b is also present on EBV-transformed B cells obtained from a patient with "Bare Lymphocyte Syndrome", that completely lack HLA class II antigens. Therefore, the Fc receptor for mIgG2b and HLA class II antigens are not identical. Since the low-affinity receptor for IgE (FcεRII; CD23) was reported to be associated at the cell surface with HLA class II antigens, we have compared both types of Fc receptor, and observed that human IgE strongly inhibits the mitogenic effect of murine IgE anti-CD3 but not of mIgG2b anti-CD3 mAb. We conclude that the human Fc receptor for mIgG2b is strongly inhibited by anti-HLA class II mAb, but is not identical to HLA class II or FcεRII.

INTRODUCTION

Receptors for the Fc moiety of IgG (FcγR) are involved in a variety of immunological processes including phagocytosis, antibody-dependent cell-mediated cytotoxicity, and the release of several mediators of inflammation. Human FcγR are heterogeneous, and at least three classes of FcγR can be distinguished (1,2). Human FcγR can also cross-react with murine (m) IgG. The mitogenic response of human T cells to murine anti-CD3 monoclonal antibody (mAb) requires the interaction of the Fc moiety of the antibody with FcγR present on human monocytes. FcγRI can bind mIgG2a, whereas the polymorphic FcγRII can, in approximately 70% of normal individuals ("mIgG1 high-responders", mIgG1-HR) interact with mIgG1 (3-6). We have recently described a human Fc receptor that is cross-reactive with mIgG2b mAb. This receptor is also polymorphic: mononuclear cells from only 3% of normal individuals ("mIgG2b high-responders", mIgG2b-HR) exhibit a mitogenic response to mIgG2b anti-CD3 mAb (7). The FcγR for mIgG2b could be demonstrated on monocytes and on EBV-transformed B cells from mIgG2b-HR, but not on cells obtained from mIgG2b low-responders (mIgG2b-LR). This Fc receptor appeared to be different from FcγRII since anti-FcγRII mAb (that completely inhibited the mIgG1 anti-CD3 mAb induced proliferative response) caused no inhibition of the mIgG2b anti-CD3 mAb induced T cell proliferation (7). Also, the release of IL2 or IFNγ, which accompanies mIgG2a or mIgG1 anti-CD3 mAb induced T cell proliferation, was not observed when T cell proliferation was induced by mIgG2b anti-CD3 mAb (8).

We now report on the strong inhibitory effect of anti-HLA class II mAb on the functioning of the FcγR for mIgG2b. In early studies on murine FcγR, a close association between Fc receptors and MHC class II antigens was found. Antibodies against MHC class II (but not anti-MHC class I) completely inhibited the binding of aggregated immunoglobulins to murine B lymphocytes, and this inhibition was not Fc-mediated (9). In studies on human cells, it has been reported that anti-HLA class II antibodies strongly inhibit the FcγRI-dependent proliferation of human mononuclear cells induced by mIgG2a anti-CD3 mAb (10), but other investigators found no such inhibition (11), or inhibition by some but not all anti-HLA-DR antibodies (12). In view of the strong inhibition of the FcγR for mIgG2b by anti-HLA class II mAb, we investigated whether expression of HLA class II antigen is necessary for the

expression of this Fc receptor, and whether HLA class II antigen is perhaps a part of (or even identical to) this receptor for mIgG2b. Furthermore, since it has been published that the human low-affinity FcR for IgE (FcϵRII; CD23) is spatially associated with HLA-DR antigens (13,14), we have also investigated whether the receptor for mIgG2b might be identical to this IgE receptor.

MATERIALS AND METHODS

Antibodies: Myeloma protein MOPC 195 (mIgG2b) was purchased from Litton Bionetics (Kensington, MD, USA). Hybridoma cell lines producing anti-HLA class I mAb W6/32 (mIgG2a), or anti-HLA class II mAb L227 (mIgG1), or anti-HLA class II mAb IVA 12 (mIgG1), were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). The anti-HLA class II mAb anti-HLA-DP (clone B7/21, mIgG1), anti-HLA-DQ (clone SK10, mIgG1) and anti-HLA-DR (clone L243, mIgG2a) were obtained from Becton Dickinson (Mountain View, CA, USA), whereas mAb OKDR (mIgG2a) was from Ortho Diagnostics (Raritan, NJ, USA). These mAb were dialysed extensively before use in order to remove the azide. mAb 2-2-1 (mIgG2a), directed against mouse MHC class II (15), as well as the anti-HLA class II mAb 12-10 (mIgG2a) and 12-13 (mIgG2b) were developed in our laboratory. $F(ab')_2$ fragments from mAb 12-10 were obtained by pepsin cleavage, by using the Immunopure $F(ab')_2$ preparation kit (Pierce, Rockford, Il, USA). The purity of these $F(ab')_2$ fragments was tested in a functional assay by measuring their inhibitory effect on mIgG2a anti-CD3 induced T cell proliferation (see Results). Human IgE (hIgE) serum (7800 U/ml) was from Behring (Marburg, Germany). U266, a hIgE producing myeloma cell line was obtained from ATCC. Anti-CD3 mAb WT32 (mIgG2a) (3) and anti-TcR mAb WT31 (mIgG1) (3,16) were developed in our laboratory. mAb WT31-2b (mIgG2b) is an isotype switch-variant obtained from WT31. Anti-CD3 mAb SPV-T3a (mIgG2b) (17) was a kind gift from Dr. H. Spits (DNAX, Palo Alto, CA, USA), and mIgE anti-CD3 mAb CLB-T3/4.E (18) was kindly provided by Dr. R. van Lier, CLB, Amsterdam, The Netherlands. Murine IgG2b anti-glycophorin A mAb is an isotype switch-variant of the mIgG1 mAb that we have previously used to study the polymorphic binding of mIgG1 to human FcγRII (4), and was a kind gift from Dr. L. Aarden, CLB, Amsterdam, The Netherlands. $F(ab')_2$ fragments from anti-glycophorin A were prepared by pepsin cleavage.

Cells and cell lines: B lymphoblastoid cell line SB was obtained from ATCC. PBMC were obtained from buffy coats or from leucocytes isolated by cytapheresis from healthy donors by centrifugation on a Ficoll-Hypaque gradient, density 1.077 g/ml (Pharmacia, Uppsala, Sweden). Lymphocytes were purified from PBMC by counterflow centrifugation. Monocytes were also isolated from PBMC by counterflow centrifugation (19) and were >90% pure, as judged by staining with May-Grünwald-Giemsa and non-specific esterase. PBMC and monocytes were used directly or cryopreserved and used later. The recovery and viability

of the cryopreserved cells was >70% and >95%, respectively. T lymphocytes were isolated by rosetting with sheep RBC treated with aminoethyl-isothiouronium bromide (20). The purified T lymphocytes were cryopreserved and used later in the T cell proliferation assay. EBV-transformed B cells were obtained from mIgG2b high-responders or low-responders, as described previously (7). PBMC, monocytes and EBV-transformed B cells were given the same identification number as the individual from which they were derived. EBV-transformed B cells (*Mycoplasma*-negative), hybridoma cell lines, and cell line SB were cultured in culture medium consisting of RPMI-1640 medium containing Hepes and sodium bicarbonate (Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT, USA), 2 mM glutamine, 1 mM sodium pyruvate, 50 μg/ml streptomycin and 50 IU/ml penicillin. An HLA class II-negative EBV-transformed B cell line obtained from a patient with "Bare Lymphocyte Syndrome" (patient THF, ref. 21)) was kindly provided by Dr. P. van den Elsen (Department of Immunohaematology & Bloodbank, Leiden, The Netherlands).

Immunoprecipitation: Cell surface iodination of B lymphoblastoid SB cells was performed by the lactoperoxidase method. Cell lysates obtained by solubilization in NP-40 lysis buffer were clarified by centrifugation and precleared with Protein A Sepharose 4B CL (Pharmacia LKB Biotechnology, Uppsala, Sweden) and with goat anti-mouse IgG (Cappel, Malvern, PA, USA) coupled to Sepharose 4B. Aliquots of lysate were treated by three absorption cycles with anti-HLA class II mAb OKDR or mAb 12-10, bound to goat anti-mouse IgG on Sepharose 4B. Other aliquots of the lysate were left untreated or were absorbed with a non-relevant mAb (2-2-1), bound to goat anti-mouse IgG-Sepharose. Immunoprecipitation was performed on aliquots of untreated or absorbed lysates by incubating with mAb 2-2-1, mAb 12-10, mAb OKDR, or mAb W6/32 (anti-HLA class I) bound to goat anti-mouse IgG-Sepharose. The immunoprecipitates were washed before analysis on 10% SDS-PAGE under reducing conditions.

T cell proliferation assay: T cell proliferation was measured by a standard [^{3}H]-thymidine incorporation assay (3) using either PBMC, or purified autologous T cells plus autologous accessory cells (monocytes or irradiated (60 Gy) EBV-transformed B cells). Briefly, 10^5 PBMC or 10^5 purified T cells plus 2 x 10^4 accessory cells were incubated in the culture medium described above in the presence of the following anti-TcR/CD3 mAb: WT31, 100 ng/ml; WT32, 50 ng/ml; SPV-T3a, 1 μg/ml; or mIgE anti-CD3, 1 μg/ml, for 72 hr at 37 °C in a humidified atmosphere of 5% CO_2 in U-bottom microtitre wells. During the last 18 hr of the incubation period [^{3}H]-thymidine (1.85 x 10^4 Bq/well) was present. All proliferation experiments were performed in triplicate. mAb tested for inhibitory effect were preincubated (10 μg/ml, 30 min. at 20 °C) with the cells before the addition of anti-CD3 mAb. The percentage inhibition was calculated from the number of c.p.m. obtained in the presence and absence of an inhibiting mAb.

EA-mIgG2b rosetting assay: Human RBC were sensitized with anti-glycophorin A mAb of mIgG2b isotype (EA-mIgG2b), or with $F(ab')_2$ fragments of anti-glycophorin A mAb as a negative control. EBV-transformed B cells (2 x 10^6/ml) were incubated with 0.5% (vol/vol) sensitized human RBC for 1 hr at room temperature (20 °C) after centrifugation for 4 min. at 20 °C (20 x *g*). The percentage of rosettes (≥ 3 RBC bound) was scored microscopically. Rosetting was carried out at normal ionic strength (PBS) or at low ionic strength (a mixture of one part of PBS and two parts of a 5% glucose suspension, pH = 7.4) (22). In some experiments EBV-transformed B cells were preincubated (30 min. at 4 °C) with anti-HLA class II mAb or mIgG2b anti-TcR mAb (WT31-2b), and washed twice before rosetting.

RESULTS

Anti-HLA class II mAb inhibits mIgG2b anti-CD3 induced T cell proliferation

As described earlier, in mIgG2b-HR individuals no inhibition of the mIgG2b anti-CD3 induced mitogenic response was found when PBMC had been preincubated with anti-FcγRII mAb (7). Remarkably, however, a strong inhibition of this mitogenic response was observed when the cells had been preincubated with mAb 12-10 or 12-13.

Table 1.

Anti-HLA class II antibodies inhibit mIgG2b anti-CD3 mAb induced T cell proliferation

mAb	Subclass	Antigen	% inhibition*	*n*
MOPC 195	mIgG2b	--	31.5 ± 12.0	2
W6/32	mIgG2a	HLA class I	18.4 ± 16.6	5
L227	mIgG1	HLA class II	99.8 ± 0.5	5
IVA 12	mIgG1	HLA class II	99.4 ± 0.9	5
12-13	mIgG2b	HLA class II	96.2 ± 5.6	6

* PBMC from mIgG2b high-responder individuals were preincubated with 10 μg/ml of the indicated antibodies, and stimulated with 1 μg/ml of mIgG2b anti-CD3 mAb. Results are expressed as the percentage inhibition of [^{3}H]-thymidine uptake after three days of culture.

Tissue distribution studies suggested that these antibodies, prepared in our laboratory, were directed against HLA-DR. Sequential immunoprecipitation studies performed with mAb 12-10 and anti-HLA-DR mAb OKDR clearly demonstrated that 12-10 indeed binds to HLA-DR. Similar studies comparing mAb 12-10 and 12-13 have shown that 12-13 is also specific for HLA-DR (data not shown). Table 1 illustrates the strong inhibitory effect of mAb 12-13, and of two other anti-HLA-DR mAb, on the T cell proliferation induced by mIgG2b anti-CD3 mAb. Three other anti-HLA class II mAb (OKDR, anti-HLA-DR, and anti-HLA-DP) also caused essentially complete inhibition of mIgG2b anti-CD3 mAb induced T cell proliferation (90-100% inhibition, four experiments). By contrast, mAb anti-HLA class I (W6/32) or mIgG2b myeloma antibody induced only marginal inhibition.

Inhibition by anti-HLA class II mAb is not Fc-mediated

Since T cell proliferation induced by anti-CD3 mAb is Fc-dependent, the inhibitory effect of anti-HLA-DR mAb might be Fc-mediated. The fact that anti-HLA class II mAb of all subclasses tested (mIgG1, mIgG2a, and mIgG2b) all caused complete inhibition of the mIgG2b anti-CD3 induced mitogenic response already argues against this explanation. In order to further investigate the Fc-dependency of the inhibitory effect, we prepared $F(ab')_2$ fragments of anti-HLA-DR mAb 12-10.

Table 2.

Inhibition of mIgG2b anti-CD3 mAb induced T cell proliferation by anti-HLA class II mAb is Fc-independent

mAb	Inhibition* of mIgG2a response	*n*	Inhibition* of mIgG2b response	*n*
12-10 IgG	100 ± 0	4	94.4 ± 4.5	14
12-10 $F(ab')_2$	1.0 ± 9.6	5	95.6 ± 9.0	8

* PBMC from mIgG2b-high-responder individuals were preincubated with 10 μg/ml of the indicated antibody or $F(ab')_2$ fragments, and stimulated with mIgG2a or mIgG2b anti-CD3 mAb. Results are expressed as the percentage inhibition of [^{3}H]-thymidine uptake after three days of culture.

Intact (mIgG2a) mAb 12-10 caused complete inhibition of the mitogenic effect of both mIgG2a and mIgG2b anti-CD3 mAb. $F(ab')_2$ fragments of mAb 12-10 did not cause any inhibition of the mIgG2a anti-CD3 mAb induced T cell proliferation, indicating that these $F(ab')_2$ fragments are completely devoid of undigested mIgG2a antibody (Table 2). By contrast, when PBMC from a mIgG2b high-responder were preincubated with $F(ab')_2$ fragments of mAb 12-10 and stimulated with mIgG2b anti-CD3 mAb, a complete inhibition of the T cell response was still observed (Table 2). Significant inhibition of mIgG2b anti-CD3 induced T cell proliferation already occurred at low concentrations of $F(ab')_2$ fragments of mAb 12-10 (73% inhibition at 50 ng/ml, Figure 1). FACS analysis showed that approximately half-maximal saturation of HLA-DR was obtained at this mAb concentration of 50 ng/ml (data not shown). mAb 12-10, 12-13, or $F(ab')_2$ fragments of mAb 12-10 caused a similar, strong inhibition of mIgG2b anti-CD3 mAb induced T cell proliferation when purified monocytes or EBV-transformed B lymphocytes from mIgG2b high-responders together with autologous T cells were used instead of PBMC (Table 3).

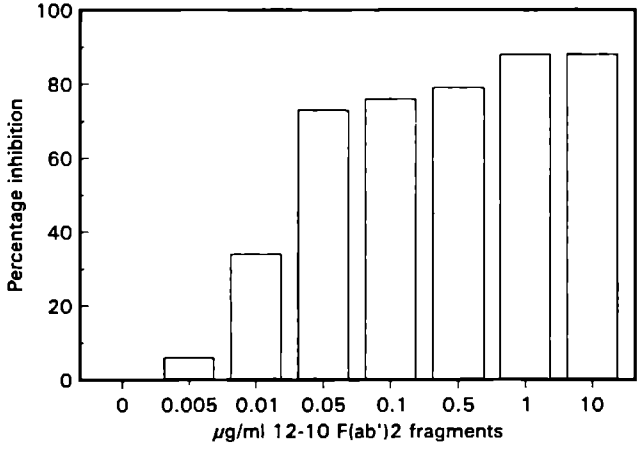

Figure 1.

Inhibition of mIgG2b anti-CD3 mAb induced T cell proliferation by $F(ab')_2$ fragments of anti-HLA class II mAb. PBMC from a mIgG2b high-responder individual were preincubated with increasing concentrations of $F(ab')_2$ fragments of mAb 12-10, and stimulated with mIgG2b anti-CD3 mAb (1 μg/ml). Results are expressed as percentage inhibition of [^{3}H]-thymidine uptake.

Table 3.

Inhibition by anti-HLA class II mAb of mIgG2b anti-CD3 mAb induced T cell proliferation in the presence of purified autologous accessory cells

Cell suspension	Donor no.	12-10	12-13	12-10 $F(ab')_2$
PBMC	1	97 ± 4*	100 ± 1	99
	5	90 ± 5	91 ± 9	89 ± 14
Monocytes and T cells	1	96 ± 2	97 ± 1	98 ± 1
	2	93 ± 2	96 ± 0	98 ± 1
	3	92	100	99
EBV-B cells and T cells	1	88 ± 3	81 ± 8[a]	72 ± 3
	2	78 ± 0	72 ± 8[a]	71 ± 3
	3	77 ± 10	74[a]	68 ± 19

* Results are expressed as percentage inhibition of T cell proliferation. Cell suspensions (from mIgG2b-HR individuals) were preincubated with 10 μg/ml of anti-HLA class II mAb (or $F(ab')_2$ fragments) and stimulated with 1 μg/ml of mIgG2b anti-CD3 mAb.
a) Preincubation with 2 μg/ml 12-13 (instead of 10 μg/ml).

Anti-HLA class II also inhibits EA-mIgG2b rosetting

EA-rosetting can be used to demonstrate the presence of Fc receptors, and we have previously shown that EBV-transformed B cells from mIgG2b-HR can form EA-mIgG2b rosettes (7). We observed no such rosettes when $F(ab')_2$ fragments of the anti-glycophorin A mAb were used to sensitize the erythrocytes. This finding confirms the Fc dependency of this EA-rosetting technique. EA-mIgG2b rosetting of EBV-transformed B cells from mIgG2b-HR (carried out at normal ionic strength) was almost completely blocked by anti-HLA class II mAb (Figure 2). When EA-mIgG2b rosetting was performed at low ionic strength, the percentage of rosetting was higher than at normal ionic strength, as reported previously (7), but inhibition by anti-HLA class II was still almost complete (data not shown). Both anti-HLA-DP and anti-HLA-DR mAb (or $F(ab')_2$ fragments thereof) were inhibitory (Figure 2). No inhibition was observed with anti-HLA-DQ mAb, probably as a result of the low expression

of HLA-DQ on these EBV-transformed B cells that was measured in immunofluorescence studies (data not shown). EA-mIgG2b rosetting was not inhibited when EBV-transformed B cells were preincubated with a control mAb of mIgG2b subclass (WT31-2b).

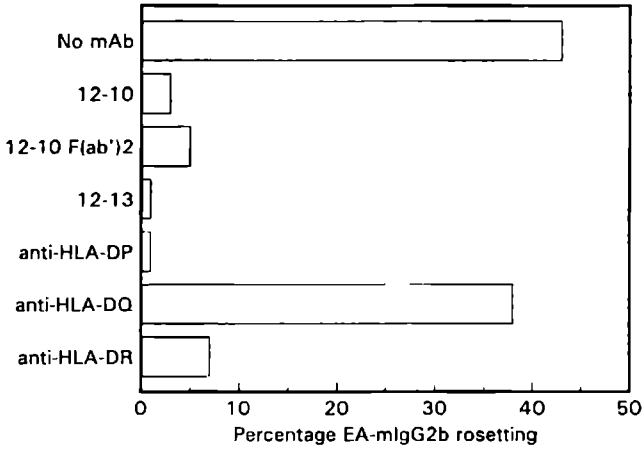

Figure 2.

Inhibition of EA-mIgG2b rosetting of EBV-transformed B cells from a mIgG2b-HR by anti-HLA class II mAb or $F(ab')_2$ fragments thereof. Cells were preincubated with the indicated antibodies, and rosetting was then performed at normal ionic strength. Values given are from a representative experiment. Similar inhibition patterns were observed with cells obtained from four mIgG2b-HR individuals. Each inhibitory antibody was tested two to four times.

EA-mIgG2b rosetting of HLA class II-negative EBV-transformed B cells

One conceivable explanation for the observed inhibition by anti-HLA class II mAb (both in the T cell proliferation assay and in the EA-mIgG2b rosetting) would be provided by identity of the Fc receptor for mIgG2b and HLA class II antigens, i.e. direct binding of mIgG2b to HLA class II. We have, however, observed EA-mIgG2b rosetting (37 - 49% rosetting observed in five rosetting experiments performed at low ionic strength) of EBV-transformed B cells from a patient suffering from the "Bare Lymphocyte Syndrome". This patient was reported to be completely negative with respect to HLA class II antigen expression (21), and we could confirm the absence of HLA class II on the

EBV-transformed B cells using immunofluorescence (data not shown). Furthermore, the EBV-transformed B cells from this patient were able to support the mitogenic response (of heterologous T cells) to mIgG2b anti-CD3 mAb. As expected, EA-mIgG2b rosetting of these EBV-transformed B cells was not inhibited by anti-HLA class II mAb (data not shown). These results indicate that expression of HLA class II is not required for the binding of mIgG2b antibody, and that HLA class II antigen and the Fc receptor for mIgG2b are separate structures on the cell membrane of EBV-transformed B cells from mIgG2b-HR individuals.

Fc receptor for mIgG2b appears different from FcεRII (CD23)

As mentioned in the Introduction, the human low-affinity receptor for IgE (FcεRII; CD23) is spatially associated with HLA-DR antigens. We therefore tested a possible relationship between this FcεRII and the Fc receptor for mIgG2b. As shown in Figure 3, the mitogenic response of mononuclear cells to mIgE anti-CD3 mAb is strongly inhibited by human serum containing a high concentration of IgE (81% inhibition at 78 U/ml).

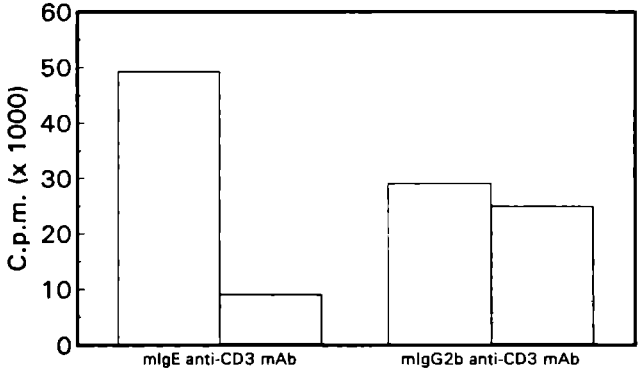

Figure 3.

Comparison of the effect of human IgE on T cell proliferation induced by mIgE anti-CD3 or mIgG2b anti-CD3 mAb. PBMC from a mIgG2b-HR individual were stimulated with 100 ng/ml of anti-CD3 mAb, in the absence (open bars) or presence (shaded bars) of human IgE serum (78 U/ml).

By contrast, the T cell proliferation induced by mIgG2b anti-CD3 mAb was hardly affected (14% inhibition) by human IgE. A similar inhibition pattern (66% inhibition of mIgE response, 18% inhibition of mIgG2b response at 1000 U/ml) was obtained when human IgE produced by a myeloma cell line (U266) was used instead of serum human IgE. Normal human serum did not inhibit the mitogenic effect of mIgE or mIgG2b anti-CD3 mAb (data not shown).

DISCUSSION

In a previous publication we have presented evidence for the existence of a polymorphic Fc receptor that can interact with mIgG2b. Anti-CD3 mAb of mIgG2b subclass was mitogenic for mononuclear cells from mIgG2b-HR individuals, and EBV-transformed B cells from such individuals were able to form EA-mIgG2b rosettes. The involvement of the Fc moiety of mIgG2b in these phenomena was demonstrated by the observation that no rosettes were formed when, instead of mIgG2b, a mIgG1 mAb of the same specificity was used to sensitize the erythrocytes. Furthermore, mIgG2b but not mIgG1 anti-CD3 mAb was mitogenic for purified T cells in the presence of EBV-transformed B cells from mIgG2b-HR (7). The Fc dependency of the EA-mIgG2b rosetting was confirmed in the present study by the absence of rosettes when $F(ab')_2$ fragments of the anti-glycophorin A antibody were used for sensitization.

The purpose of the present study was to investigate the role of HLA class II antigens with respect to the human Fc receptor for mouse IgG2b. Several anti-HLA class II mAb completely inhibited the functioning of this Fc receptor in two different assays: mIgG2b anti-CD3 mAb induced T cell proliferation, and EA-mIgG2b rosetting of EBV-transformed B cells. It has been reported previously that anti-HLA class II mAb can inhibit T cell proliferation induced by mIgG2a anti-CD3 mAb (10,12) although some other anti-HLA class II mAb had no effect in this system (11,12). This inhibitory effect was Fc-independent since it was also observed with $F(ab')_2$ fragments, and both accessory cells (monocytes) and (activated) T cells appeared to be targets of the inhibition (10). The inhibition that we describe here appears to be different in several respects. First, although the anti-HLA class II mAb 12-10 inhibited the mitogenic effect of mIgG2a anti-CD3 mAb, $F(ab')_2$ fragments of 12-10 were not inhibitory in this assay but they still caused complete inhibition of mIgG2b anti-CD3 mAb

induced T cell proliferation. The inhibitory effect of $F(ab')_2$ fragments of 12-10 is therefore specific with respect to the Fc receptor for mIgG2b, since these fragments do not affect T cell proliferation induced by anti-CD3 mAb of mIgG2a subclass, which is dependent on monocyte FcγRI. It is very unlikely that in this case the inhibitory effect would be mediated by binding of the anti-HLA class II mAb to (activated) T cells. Furthermore, anti-HLA-DR mAb as well as $F(ab')_2$ fragments of 12-10 completely inhibited the formation of EA-mIgG2b rosettes. These findings strongly suggest that the inhibitory effect described here is mediated, at the level of the accessory cell, by a direct interference of anti-HLA class II mAb with the functioning of the human Fc receptor for mIgG2b.

How then can antibodies against HLA class II interfere with the binding of mIgG2b? One obvious (although not very likely) possibility is that HLA class II antigens themselves function as Fc receptor for mIgG2b. The observation that a "Bare Lymphocyte Syndrome" patient, completely lacking expression of HLA class II, is a high-responder with respect to mIgG2b appears to rule out this possibility. Furthermore, we have found no evidence for a particular HLA-DR phenotype among the different HLA class II-positive mIgG2b high-responders (data not shown). Another possibility is that HLA class II antigens are closely associated with the Fc receptor for mIgG2b, as is the case for the low-affinity Fc receptor for IgE (13,14). The inhibitory effect of anti-HLA class II mAb on both Fc receptors even suggests that these receptors might be identical. Several lines of evidence, however, argue against this hypothesis. First, mIgE anti-CD3 mAb induces T cell proliferation with mononuclear cells from all individuals (18) whereas mIgG2b anti-CD3 mAb is mitogenic with only 3% of normal individuals (7). Furthermore, in this T cell proliferation assay mIgG2b anti-CD3 mAb does not induce IL2 or IFNγ (8) whereas mIgE anti-CD3 mAb induced T cell proliferation is associated with cytokine release (L. Frenken, personal communication). The data presented in this study also argue against identity of both receptors: human IgE strongly inhibits the mitogenic effect of mIgE anti-CD3, but not of mIgG2b anti-CD3 mAb. Therefore, the Fc receptor for mIgG2b appears to be different from FcϵRII. It could, however, still be associated with HLA class II antigens on the cell surface. In an attempt to directly demonstrate such an association, we radiolabeled EBV-transformed B cells from a mIgG2b-HR individual, cross-linked the cell lysate, and performed immunoprecipitation with anti-HLA-DR mAb. We have been unsuccessful so far in immunoprecipitating an additional polypeptide apart from the HLA-DR chains, as was described for FcϵRII (13). This does not exclude the possibility,

however, that such an association does exist. Alternatively, it is conceivable that the observed inhibitory effect of anti-HLA class II mAb is not due to a physical association of HLA class II antigens and the Fc receptor for mIgG2b, but is the result of some cellular signalling process mediated by HLA class II antigen. It has been reported, for instance, that the binding of anti-HLA-DR can induce IL1 mRNA and cellular IL1 production (23). The precise nature, however, of the HLA-DR mediated signal transduction pathway that might play a role in the functioning of the Fc receptor for mIgG2b remains to be elucidated. Since strong inhibition by anti-HLA class II mAb was also observed in the rosetting assay (where the anti-HLA class II antibodies are present for only a short time, and at room temperature or below), we favour (despite the absence of biochemical proof) the explanation of a cell surface association between HLA class II antigens and the Fc receptor for mIgG2b.

The biological significance of the Fc receptor for mIgG2b on human cells is not yet known. Studies to define this receptor at the DNA level are in progress, and these studies will hopefully enable us to isolate and transfect the relevant cDNA and study the binding characteristics and biological significance of this Fc receptor in more detail.

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Proteolysis increases the Fc-mediated binding of murine IgG2b to human EBV-transformed B cells, but decreases the expression of FcγRII and FcεRII

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ABSTRACT

We have previously described a polymorphic human Fc receptor for murine IgG2b (mIgG2b). This receptor was defined by the binding of (complexed) mIgG2b to monocytes and Epstein-Barr virus (EBV)-transformed B cells. Three per cent of normal individuals were high-responders with respect to mIgG2b (mIgG2b-HR), whereas the other individuals were low-responders for mIgG2b (mIgG2b-LR). In the present study we investigated the effect of proteolytic enzymes on the Fc-mediated binding of mIgG2b to EBV-B cells. Pronase, human leucocyte elastase, and cathepsin G caused an increased binding (in an EA-rosetting assay) of mIgG2b to EBV-B cells from mIgG2b-HR, but not from mIgG2b-LR. Simultaneous immunofluorescence studies demonstrated that these proteolytic enzymes strongly reduced the expression of FcγRII and FcεRII on these cells, whereas HLA class I or HLA class II molecules were not affected. These findings strongly suggest that binding of mIgG2b is not mediated by FcγRII or FcεRII. We also studied the effect of proteolysis on mIgG2b-HR EBV-B cells from an HLA class II-negative individual. In this case EA-mIgG2b rosetting was decreased after proteolysis, suggesting that HLA class II molecules may have a role in protecting the binding site for mIgG2b against proteolytic destruction.

INTRODUCTION

Specific receptors for the Fc moiety of IgG (FcγR) are expressed on leucocytes, and play an important role in the immune defense mechanism. In inflammation, FcγR-positive cells like monocytes and neutrophils are involved in phagocytosis (1,2), release of mediators (3), degranulation (4), and antibody dependent cell-mediated cytotoxicity (ADCC) (5). Three classes of human (h) FcγR have been characterized in detail at the cellular and molecular level: FcγRI, FcγRII, and FcγRIII. They all belong to the Ig-supergene family and their genes have been mapped to chromosome 1 (5,6). Human FcγR can cross-react with murine (m) IgG as determined in an anti-CD3 monoclonal antibody (mAb) induced T cell proliferation assay. The high-affinity receptor, FcγRI (CD64), can bind monomeric mIgG2a (7). The polymorphic FcγRII (CD32), a low-affinity receptor, can interact with aggregated or complexed mIgG1 in 70% of the Caucasian individuals (mIgG1 high-responders; mIgG1-HR) (8,9).

Recently, we described a polymorphic human Fc receptor which can interact with complexed mIgG2b and which is present on monocytes and (EBV-transformed) B cells from 3% of healthy Caucasian individuals (10). The polymorphism of this receptor was defined using a T cell proliferation assay (10-12): mIgG2b anti-CD3 mAb was mitogenic for peripheral blood mononuclear cells from mIgG2b high-responder individuals (mIgG2b-HR) but not from mIgG2b low-responders (mIgG2b-LR). The presence of a human Fc receptor for mIgG2b on EBV-transformed B cells from mIgG2b-HR individuals was confirmed by EA-rosetting, using mIgG2b-sensitized erythrocytes (10). These EBV-transformed B cells do not express FcγRI or FcγRIII, but they do express an isoform of FcγRII. The FcR for mIgG2b, however, appeared to be different from FcγRII since the mitogenic response of mIgG2b anti-CD3 mAb was not inhibited by anti-FcγRII mAb (10). Furthermore, T cell proliferation induced by mIgG2b anti-CD3 mAb was not inhibited by anti-IL2 receptor mAb and no release of IL2 or IFNγ was measured, whereas T cell proliferation induced by mIgG2a or mIgG1 anti-CD3 mAb (that interacted with FcγRI and FcγRII, respectively) was always accompanied by the release of these cytokines and was inhibited significantly by anti-IL2 receptor mAb (13). The FcR for mIgG2b seemed to be associated with HLA class II molecules, since anti-HLA class II mAb (both intact IgG and $F(ab')_2$ fragments) completely inhibited the mIgG2b anti-CD3 mAb induced T cell response as well as the EA-mIgG2b

rosetting (14). The FcR for mIgG2b was, however, also expressed on EBV-B cells from an HLA class II-negative individual (14) which indicates that expression of HLA class II antigens is not an absolute requirement for binding of mIgG2b to human cells. Since the low-affinity receptor for IgE (FcεRII; CD23) is associated with HLA class II antigens (15), we included this IgE receptor in the present study.

Proteolytic enzymes can have a differential effect on different Fc receptors. The Fc receptor for IgE on the human monocytic cell line U937 was reported to be trypsin sensitive, whereas the receptor for IgG was trypsin resistant. As a matter of fact, the binding of human IgG1 was enhanced over that seen with untreated cells (16). In studies that were performed many years later, when much more was known about the different classes of Fc receptors for IgG, we could demonstrate that proteolysis had no effect on human monocyte FcγRI, whereas the affinity of human monocyte FcγRII for IgG was strongly increased by the proteolytic enzymes trypsin and pronase. The number of FcγRII molecules was not affected (17,18). Similar results were obtained with human leucocyte elastase (19). An increased EA-rosetting of human monocytes has also been observed after treating the monocytes with neuraminidase, an enzyme that has no proteolytic activity but that can remove sialic acid from sialoglycoproteins (20).

In the present study EBV-B cells from mIgG2b-HR individuals were treated with the serine proteases human leucocyte elastase or cathepsin G, both purified from human granulocytes. The percentage of EA-mIgG2b rosettes increased after proteolytic treatment, whereas the expression of FcγRII and FcεRII was strongly reduced. These results support the hypothesis that the FcR for mIgG2b is different from FcγRII (CD32) or FcεRII (CD23).

MATERIALS AND METHODS

Antibodies: Hybridoma cell lines producing anti-HLA class I mAb W6/32 (mIgG2a) or HB118 (mIgG1) or anti-HLA class II mAb L227 (mIgG1) or IVA 12 (mIgG1) were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). Anti-HLA class II mAb 12-10 (mIgG2a) was developed in our laboratory (14). Anti-CD20 mAb B1 (mIgG2a) was from Coulter Immunology (Luton, U.K.). Anti-FcγRII mAb KB61 (mIgG1) (21), 41H.16 (mIgG2a) (22) and KuFc79 (mIgG2b) (23) were kindly provided by Drs. D.Y. Mason (John Radcliffe Hospital, Oxford, U.K.), Th.F. Zipf (Anderson Hospital and Tumor

Institute, Houston, TX, USA) and T. Mohanakumar (Washington University School of Medicine, Department of Surgery, St. Louis, MI, USA), respectively. Anti-FcγRII mAb IOM32 (clone 2E1; mIgG2a) (24) and anti-FcεRII mAb IOB8 (clone 9P25; mIgG1) were obtained from Immunotech SA (Marseille, France). Anti-FcεRII mAb Tü1 (mIgG1) was from Biotest AG (Dreieich, Germany). The anti-FcεRII mAb 9P25 was kindly provided by Dr. J. Banchereau (UNICET, Dardilly, France) (25). Anti-glycophorin A mAb of the mIgG2b or mIgG1 isotype were a kind gift from Dr. L.A. Aarden (CLB, Amsterdam, The Netherlands). MAb anti-transferrin receptor (CD71) was obtained from Becton-Dickinson (Mountain View, CA, USA).

Cells: EBV-transformed B cells were obtained from healthy HLA class II-positive (≥ 95% positive cells) mIgG2b-HR or mIgG2b-LR individuals, as described previously (10). Only EBV-B cells that were *Mycoplasma*-negative as tested in a Gen-Probe Mycoplasma Tissue Culture II detection system (10) were used. The EBV-B cells were cultured in RPMI-1640 medium (Dutch modification) containing Hepes and sodium bicarbonate (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated foetal calf serum (FCS; Hyclone, Logan, UT, USA), 2 mM glutamine, 1 mM sodium pyruvate, 50 μg/ml streptomycin and 50 IU/ml penicillin in a humidified atmosphere of 5% CO_2 at 37 °C.

HLA class II-negative EBV-B cells obtained from patients suffering from the "Bare Lymphocyte Syndrome", e.g. patient THF (26,27) were kindly provided by Dr. P. van den Elsen (Department of Immunohaematology & Bloodbank, Leiden, The Netherlands).

Reagents: The serine proteases human leucocyte elastase and cathepsin G purified from azurophilic granules of human neutrophils were kindly provided by L. Joosten (Department of Rheumatology, University Hospital Nijmegen, Nijmegen, The Netherlands). Pronase isolated from *Streptomyces griseus* was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). The reagents neuraminidase (type V, isolated from *Clostridium perfringens*); pancreatic elastase (type IV, from porcine pancreas) and PMSF (phenylmethylsulfonyl fluoride, an irreversible inhibitor of serine proteases) were from Sigma Diagnostics (St. Louis, MO, USA).

Treatment of EBV-B cells with proteolytic enzymes or neuraminidase: EBV-B cells (5 x 10^6 per ml) in serum-free culture medium were treated with increasing concentrations (see Results) of proteolytic enzymes and/or protease inhibitor or neuraminidase and incubated for 30 min. at 37 °C. Ice-cold culture medium containing 10% FCS was added to the cell suspension and incubated for 15 min. at 0 °C to inactivate enzymatic activity. Cells were washed twice in PBS (phosphate buffered saline; pH = 7.4) alone or PBS containing 1% bovine serum albumine (PBS 1% BSA) and used for EA-mIgG2b rosetting at normal ionic strength or immunofluorescence studies, respectively, as described below. Loss of cells after proteolytic treatment with 100 μg/ml human leucocyte elastase or cathepsin G and washing

was 30 ± 9 % (n = 3) and 17 ± 7% (n = 3), respectively. After treatment with neuraminidase (100 μg/ml) and washing, 17 ± 3 % (n = 5) of the cells were lost.

EA-mIgG rosetting assay: EA-mIgG2b or EA-mIgG1 rosetting assay at normal ionic strength was performed as described (10). Human erythrocytes (HRBC) were maximally sensitized with anti-glycophorin A mAb of mIgG2b (EA-mIgG2b) or mIgG1 (EA-mIgG1) isotype. Briefly, EBV-B cells (2 x 10^6 /ml) were incubated with 0.5% (vol/vol) sensitized HRBC in PBS, centrifugated for 4 min. at 20 °C (20 x *g*) and incubated for 1 hr at 20 °C (room temperature). The percentage of rosettes (≥ 3 HRBC bound) was scored microscopically.

Immunofluorescence assay: Immunofluorescence studies were carried out using standard procedures. Briefly, 4 - 5 x 10^5 cells were incubated with mAb for 30 min. at 0 °C, washed in PBS 1% BSA and 0.1% sodium azide, labeled with FITC-conjugated $F(ab')_2$ fragments of sheep anti-mouse IgG, heavy and light chain specific (Cappel, Malvern, PA, USA) and 5000 cells from each sample were analysed in a Coulter Epics flow cytometer.

RESULTS

EA-mIgG2b rosetting increases after treatment with proteolytic enzymes

The effect of the serine proteases leucocyte elastase and cathepsin G on the EA-mIgG2b rosetting of HLA class II-positive EBV-B cells from mIgG2b-HR and mIgG2b-LR individuals was studied, using varying concentrations (0 - 200 μg/ml) of proteolytic enzymes. A two-fold increase of EA-mIgG2b rosetting was measured when EBV-B cells from mIgG2b-HR were incubated with a concentration of 100 μg/ml of leucocyte elastase or cathepsin G (Figure 1A). Whereas with EBV-B cells from mIgG2b-HR individuals an increased EA-mIgG2b rosetting was observed after treatment with the serine proteases pronase, leucocyte elastase and cathepsin G, no EA-mIgG2b rosetting was induced in EBV-B cells from mIgG2b-LR individuals (Table 1). The increase of EA-mIgG2b rosetting in EBV-B cells from mIgG2b-HR individuals induced by cathepsin G was completely inhibited by PMSF, an irreversible inhibitor of serine proteases. PMSF alone had no effect on the percentage of EA-mIgG2b rosettes (Table 1). Treatment of EBV-B cells with increasing concentrations of pancreatic elastase (up to 200 μg/ml) had no effect on EA-mIgG2b rosetting (data not shown). No induction of EA-mIgG1 rosetting was observed in EBV-B

cells from mIgG2b-HR or -LR individuals treated with leucocyte elastase, cathepsin G or pronase (data not shown).

Table 1.

EA-mIgG2b rosetting of EBV-B cells from mIgG2b-HR and mIgG2b-LR individuals after proteolytic treatment with pronase, leucocyte elastase, cathepsin G and/or PMSF

	mIgG2b high-responder	mIgG2b low-responder
Control	35 ± 2	0
Pronase	56 ± 4	0
Leucocyte elastase	51 ± 6	0
Cathepsin G	69 ± 6	0
Cathepsin G + PMSF	31	0
PMSF	33 ± 4	0

HLA class II-positive EBV-B cells were incubated in the absence or presence of serine proteases pronase (250 μg/ml), leucocyte elastase (100 μg/ml) or cathepsin G (100 μg/ml) and/or the serine protease inhibitor PMSF (1 mM) and tested in the EA-mIgG2b rosetting assay. Results are presented as the percentage of rosetting cells, and are mean values from one to three experiments.

Proteolytic treatment strongly reduces FcγRII expression

In immunofluorescence studies, a strong reduction of FcγRII expression was measured when EBV-B cells from mIgG2b-HR individuals were incubated with increasing concentrations of leucocyte elastase or cathepsin G (Figure 1B). Results presented were obtained with anti-FcγRII mAb KB61 (Figure 1B), but similar results were obtained with anti-FcγRII mAb KuFc79, 2E1, and 41H.16 (data not shown). Whereas leucocyte elastase, cathepsin G, and pronase strongly reduced the expression of FcγRII, they had no effect on the expression of HLA class I or HLA class II molecules (Table 2). Similar results as shown in Table 2 were obtained with anti-HLA class I mAb HB118, or anti-HLA class II mAb L227 and IVA 12. Proteolysis also caused a reduced expression of

FcγRII (but not HLA class I or HLA class II) on EBV-B cells from mIgG2b-LR individuals (data not shown).

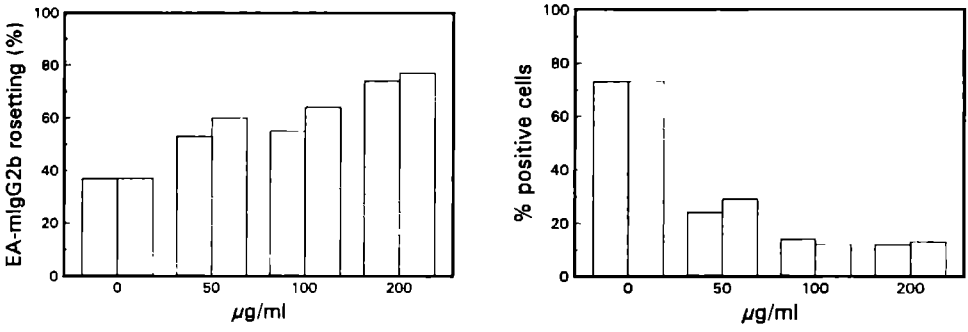

Figure 1. A B

The percentage of EA-mIgG2b rosettes (A) of HLA class II-positive EBV-B cells from a mIgG2b-HR individual was determined after treatment (30 min. at 37 °C) with increasing concentrations (0 - 200 μg/ml) of leucocyte elastase (shaded bars) or cathepsin G (open bars). Simultaneously, FcγRII expression was measured as the percentage of positively stained cells with anti-FcγRII mAb KB61 (B). Results presented are representative for a total of six experiments performed with EBV-B cells from three different mIgG2b-HR individuals.

Proteolysis abolishes expression of FcεRII

The low-affinity FcR for IgE, FcεRII/CD23 is expressed on EBV-B cells. Since this receptor is spatially associated with HLA class II molecules (15), as also seems to be the case with the Fc receptor for mIgG2b, the expression of FcεRII was also investigated before and after proteolytic treatment. Pronase, leucocyte elastase or cathepsin G caused a complete disappearance of the expression of FcεRII as measured by the anti-CD23 mAb IOB8 (Table 2). Similar results were measured with anti-FcεRII mAb Tü1: a reduction from 97% (before proteolysis) to 3% positively stained cells (after proteolysis). The same destructive effect of proteolysis on FcεRII expression was observed with EBV-B cells from mIgG2b-LR individuals (data not shown). No effect was observed on the expression of the B cell antigen CD20: more than 99% of anti-CD20 mAb B1-positively stained cells were measured both before and after proteolytic treatment.

Table 2.

Antigen expression on HLA class II-positive EBV-B cells from a mIgG2b-high-responder individual before and after proteolysis

Antigen	Control	Pronase	Leucocyte elastase	Cathepsin G
FcγRII	83 ± 7	10 ± 5	4 ± 1	5
FcεRII	98 ± 2	2 ± 1	1	3 ± 2
HLA class I	99 ± 1	99 ± 1	99 ± 1	99
HLA class II	98 ± 1	86 ± 8	96 ± 2	97 ± 2

EBV-B cells were incubated in the absence or presence of pronase (250 μg/ml), leucocyte elastase (100 μg/ml) or cathepsin G (100 μg/ml). The antigen expression was measured by anti-FcγRII mAb KB61 (n = 1 - 4); anti-FcεRII mAb IOB8 (n = 1 - 2); anti-HLA class I mAb W6/32 (n = 1 - 4); and anti-HLA class II mAb 12-10 (n = 2 - 3). Results are expressed as the percentage (mean ± SD) of positively stained cells in immunofluorescence.

Proteolytic effects on HLA class II-negative EBV-B cells

Recently we have reported that the FcR for mIgG2b and HLA class II antigens are separate molecules since HLA class II-negative EBV-B cells (from a patient suffering from the "Bare Lymphocyte Syndrome") are able to form EA-mIgG2b rosettes (14). Surprisingly, proteolytic treatment of these EBV-B cells caused a reduction of the percentage of EA-mIgG2b rosettes (Figure 2A), in contrast to the increased rosetting observed with HLA class II-positive EBV-B cells. Similar to the results obtained with HLA class II-positive EBV-B cells, the expression of FcγRII was strongly reduced after proteolytic treatment (Figure 2B), and FcεRII expression was completely abrogated (the percentage of CD23-positive cells was reduced from 96% before proteolytic treatment to less than 3% after proteolysis). HLA class II antigens were absent in these cells, and expression of HLA class I antigens was not affected by proteolysis: the percentage of HLA class I-positive cells remained more than 97%.

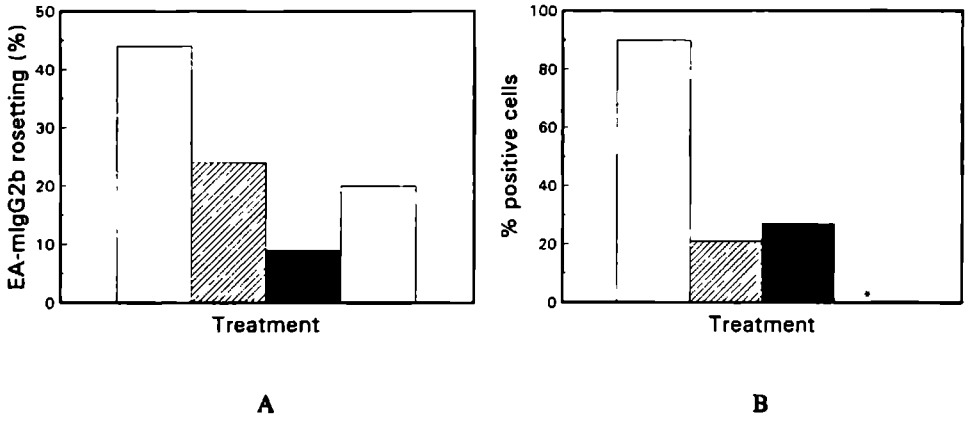

Figure 2.

The percentage of EA-mIgG2b rosettes (A) of HLA class II-negative EBV-B cells from a mIgG2b-HR individual was measured in the absence (open bar) or in the presence of 250 μg/ml pronase (hatched bars), 100 μg/ml leucocyte elastase (black bars) or cathepsin G (shaded bar). Simultaneously, FcγRII expression was determined as the percentage of positively stained cells with anti-FcγRII mAb KB61 (B). The experiment was repeated twice with comparable results. * Not determined.

Neuraminidase affects EA-mIgG2b rosetting but not FcR expression

Reduction of the negative charge of EBV-B cells might result in better adhesion of (sensitized) erythrocytes, and an increased percentage of EA-rosettes. Therefore, we tested the effect of neuraminidase (an enzyme that removes sialic acids from cell surface molecules) on EA-mIgG2b rosetting and FcR expression on EBV-B cells. EA-mIgG2b rosetting of (HLA class II-positive or -negative) EBV-B cells from mIgG2b-HR individuals was enhanced after treatment with increasing concentrations of neuraminidase (Figure 3). No induction of EA-mIgG2b rosetting was found with EBV-B cells from mIgG2b-LR individuals (data not shown). In contrast to the effects of proteolytic enzymes, no decrease of FcγRII or FcϵRII expression was observed when EBV-B cells were treated with neuraminidase (FcγRII: 69 $\pm$ 11% before neuraminidase, 70 $\pm$ 9% after neuraminidase; FcϵRII: 98 $\pm$ 1% before, 97 $\pm$ 2% after neuraminidase; similar values were obtained with HLA class II-negative EBV-B cells).

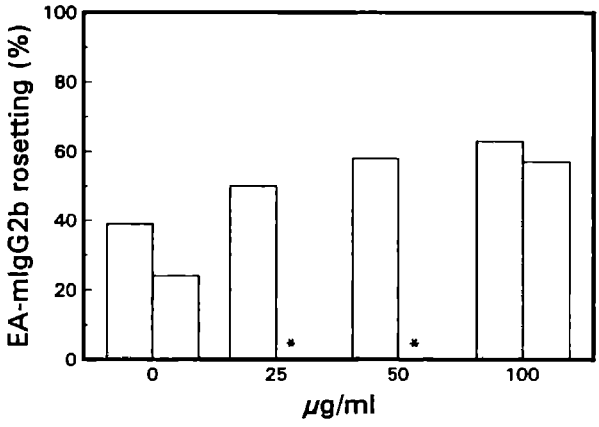

Figure 3.

EA-mIgG2b rosetting (%) of HLA class II-positive (shaded bars) or HLA class II-negative (open bars) EBV-B cells from mIgG2b-HR individuals was measured after treating the cells with increasing concentrations of neuraminidase. Results presented are representative of two to four experiments. * Not determined.

DISCUSSION

Using proteolytic enzymes, we have further characterized the polymorphic human Fc receptor that can interact with (polymeric) mIgG2b. Previously, we have reported that the induction of T cell proliferation by mIgG2b anti-CD3 mAb requires an accessory function that could be provided by either monocytes or (EBV-transformed) B cells from mIgG2b-HR individuals. These EBV-B cells do not express FcγRI or FcγRIII, but they do express an isoform of FcγRII (10). Although it is often assumed that human FcγRII is responsible for the binding of mIgG2b, there is not much experimental evidence for this assumption. In a previous study, we observed that anti-FcγRII mAb could not inhibit the mitogenic response to mIgG2b anti-CD3 mAb. Furthermore, high-responsiveness for mIgG2b occurs at a frequency (3%) that is different from the frequency of (FcγRII-mediated) mIgG1 high-responsiveness (70%), and is independent of the responder status with respect to mIgG1 (10). Another Fc receptor that is expressed on B cells is FcεRII (CD23). Since this receptor is

associated with HLA class II molecules (15), and anti-HLA class II mAb inhibits the human Fc receptor for mIgG2b (14), it seemed of interest to include FcεRII as well as FcγRII in the present investigation.

When EBV-B cells from normal (HLA class II-positive) mIgG2b-HR individuals were treated with serine proteases (pronase, human leucocyte elastase, or cathepsin G), an enhanced EA-mIgG2b rosetting was measured. An increased EA-mIgG2b rosetting was also observed when the cells were incubated with neuraminidase, indicating that neutralization of negative charge is one mechanism causing enhanced EA-rosetting. Since both leucocyte elastase and cathepsin G have a high positive charge at neutral pH (pI = 11.0 and pI = 13.0, respectively), it might be possible that the enhancing effect of these enzymes on EA-mIgG2b rosetting was related to neutralization of the negatively charged cell membrane of EBV-B cells, rather than to their proteolytic activity. Addition of the serine protease inhibitor PMSF (a low molecular weight compound that will hardly affect the charge of the enzyme) completely abolished the enhancing effect of cathepsin G. Therefore we conclude that the proteolytic activity of the serine proteases (and not their positive charge) is responsible for the enhancement of EA-mIgG2b rosetting. A remarkable detail is that, whereas leucocyte elastase could enhance EA-mIgG2b rosetting, pancreatic elastase was unable to do so. A similar observation was made previously when the effect of proteolytic enzymes on EA-mIgG1 rosetting of human myeloid cell lines was studied (19). This may be due to differences in the fine specifity of these two elastase enzymes (28). Although leucocyte elastase and several other proteases increased the EA-mIgG2b rosetting of mIgG2b-HR EBV-B cells, they did not induce EA-mIgG2b rosetting of cells obtained from mIgG2b-LR individuals. This is in contrast to the results obtained with EA-mIgG1 rosetting: when human monocytes from mIgG1-LR individuals were treated with proteolytic enzymes, FcγRII-mediated EA-mIgG1 rosetting was induced as a consequence of increased affinity of the receptor (17). Furthermore, we never observed EA-mIgG1 rosetting with EBV-B cells either before or after proteolysis. This latter finding may be related to the expression of different isoforms of FcγRII on monocytes and B cells (5,6), but it also indicates once again that binding of mIgG2b is independent of the binding of mIgG1.

The expression of FcγRII on EBV-B cells was strongly reduced by proteases, again in contrast with monocytes where the number of FcγRII molecules was not affected (17). Proteolytic cleavage of FcγRII from the cell membrane was

recently reported to occur on activated but not on resting human B cells, apparently as a result of endogenous protease activity (29). In view of these findings it seemed of interest to determine whether EBV-B cells resemble activated or resting B cells, and whether under the experimental conditions there is release of soluble Fc receptors (which might inhibit the EA-mIgG2b rosetting). The EBV-transformed B cells express high levels of HLA-DR and CD23 (Table 2). Furthermore, the transferrin receptor was expressed on a majority of the cells (81%, 68%, and 58% of the EBV-transformed B cells obtained from two mIgG2b-HR and one mIgG2b-LR, respectively). These data suggest that the EBV-B cells resemble activated more than resting B cells. In contrast, however, to the B cells that were activated by addition of anti-IgM antibody (29), no activating stimulus was deliberately given to the EBV-transformed cells. It is an interesting question whether soluble Fc receptors are released from these EBV-B cells by (endogenous) proteases. With respect to FcγRII, the high expression of this receptor on the EBV-B cells observed after the "control" incubation (no exogenous protease added, Table 2), indicates that under the experimental conditions there is apparently no proteolytic cleavage of FcγRII by endogenous proteases. With respect to the Fc receptor for mIgG2b, the increase of EA-mIgG2b rosetting after the addition of (exogenous) protease strongly argues against proteolytic cleavage of the Fc receptor for mIgG2b. However, even if soluble Fc receptors were released, they would not interfere with the EA-mIgG2b rosetting since several washing steps were performed between the incubation of the cells and the EA-rosetting assay. Furthermore, the proteolytic treatment of the EBV-transformed cells resulted in an increase, not an inhibition of rosetting. In our experiments with exogenous proteases, we used the same anti-FcγRII mAb (KB61) that was used in the study with activated B cells (29) to evaluate cell membrane expression of FcγRII. We obtained similar results with three other anti-FcγRII mAb, which excludes the possibility that proteolysis might have only damaged one particular epitope of the receptor. The strong reduction of FcγRII expression (a small percentage of cells remained positive, but even on these cells the number of receptor molecules was strongly decreased) at the same time as EA-mIgG2b rosetting was increased, indicates that the polymorphic binding of mIgG2b was probably not mediated by FcγRII.

Proteolysis completely abolished the expression of FcϵRII on EBV-B cells. This low-affinity receptor for IgE is easily shed from the cell membrane by proteolytic cleavage (30). Although FcϵRII is associated with HLA class II molecules (15), and the Fc receptor for mIgG2b is inhibited by anti-HLA class

II mAb (14), we previously reported evidence that these receptors are probably not identical since human IgE inhibited the mitogenic response to mIgE anti-CD3 but not to mIgG2b anti-CD3 mAb (14). The present results (increase of EA-mIgG2b rosetting but disappearance of FcεRII expression) support the conclusion that binding of mIgG2b is not mediated by FcεRII.

The effect of proteolysis was apparently influenced by the presence or absence of HLA class II molecules. When EBV-B cells lacking HLA class II molecules were treated with proteases, EA-mIgG2b rosetting decreased whereas it increased with normal, HLA class II-positive cells. With both types of EBV-B cells, expression of FcγRII and FcεRII was strongly reduced by proteolysis. The discrepancy between HLA class II-positive and -negative EBV-B cells with respect to the EA-mIgG2b rosetting may indicate that HLA class II molecules are involved in stabilizing and protecting the Fc receptor binding site for mIgG2b against proteolysis in HLA class II-positive mIgG2b-HR individuals. Earlier studies on a possible relationship between Fc receptors and MHC molecules have yielded contradictory results (31,32). It is difficult to compare our findings, described here and in a recent publication (14), with the data obtained in those early studies because at the time the complex heterogeneity of Fc receptors and the existence of cell type-specific isoforms were not yet known, and Fc receptors were detected using polyclonal IgG (containing different subclasses) and sometimes cell suspensions were used that contained several different cell types.

In vivo, the serine proteases leucocyte elastase and cathepsin G are released from granulocytes during activation (e.g. at inflammatory sites). These enzymes can have opposite effects on different Fc receptors present on B cells. On the one hand, they may induce the release of soluble FcγRII or FcεRII from the B cells. On the other hand, these proteases may upregulate the Fc receptor that is cross-reactive with mIgG2b at sites of inflammation, as was previously suggested for monocyte FcγRII (18). Another intriguing possibility is that the endogenous proteolytic activity observed in activated B cells (29) could cause an upregulation of the Fc receptor for mIgG2b. Since the human ligand of the Fc receptor for mIgG2b has not yet been defined, the physiological significance of such an upregulation is not yet clear.

In conclusion, the results of this study provide more evidence that the FcR for mIgG2b on (EBV-transformed) B cells is different from FcγRII or FcεRII. The FcR binding site for mIgG2b seems to be protected and stabilized by HLA class

II molecules during proteolysis. Further molecular and functional studies are needed to characterize this human FcR for mIgG2b in more detail.

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Clinical implications of the polymorphic interaction of murine IgG2b and IgG1 with human Fc receptors

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When monoclonal antibodies (mAbs) became available as an option for immunotherapy, it soon became evident that apart from specificity and affinity, the isotype of the mAb was important for its effectiveness. Different isotypes of murine (m) immunoglobulin exhibited significant differences in their capability to interact with human effector systems. Differences in their binding to human Fc receptors for IgG (Fcγ receptors) were especially prominent. Human monocytes could perform antibody-dependent cellular cytotoxicity (ADCC) against tumour cells in the presence of murine IgG2a mAb, but only slightly or not at all with the mIgG1 or mIgG2b subclasses (1). Subsequently, it was shown that monomeric mIgG2a (and mIgG3) could bind to human monocyte Fc receptors whereas no binding occurred with monomeric mIgG1 or mIgG2b (2).

Heterogeneity of human Fcγ receptors

In recent years our knowledge of human Fcγ receptors, and of their interaction with murine immunoglobulins, has rapidly increased. Three main classes of human Fc receptors for IgG (FcγRI, FcγRII, and FcγRIII) have been defined at the molecular level. Several isoforms can be distinguished within each receptor class, and further complexity is introduced by various genetic polymorphisms (3-5). With respect to murine immunoglobulins, the mIgG2a and mIgG1 isotypes have been studied in detail, and the specificity and affinity of their interaction with human Fcγ receptors have been defined. Much less, however, was known until recently about the binding of mIgG2b to human Fc receptors. We will review here some recent data on the (polymorphic) binding of mIgG2b to human cells, and discuss the implications of the polymorphism of human Fcγ receptors for the immunotherapeutical use of mIgG1 or mIgG2b mAb. As will be illustrated below, the knowledge obtained from studies on the binding of murine immunoglobulins to human Fc receptors can also be very useful for the construction of humanized antibodies (containing human Fc moieties) for immunotherapy.

Interaction of murine IgG2b with human Fc receptors

In studies employing human cell lines, binding of mIgG2b was only observed when the antibody was heat-aggregated, and even then special experimental conditions (low ionic strength) were required. In these experiments, the binding of polymeric mIgG2b was not mediated by FcγRI. There were some indications (but no conclusive evidence) for the involvement of FcγRII (6). Indirect evidence for Fc-mediated, polymorphic binding of mIgG2b to human cells was obtained in T cell proliferation assays using mIgG2b anti-CD3 mAb. We had previously demonstrated (using mIgG1 anti-CD3 mAb) that induction of T cell proliferation by anti-CD3 mAb requires the interaction of the Fc moiety of the antibody with Fc receptors on accessory cells, e.g. monocytes. The mitogenic response of peripheral blood mononuclear cells (PBMC) to mIgG1 was polymorphic: 70% of normal individuals were "mIgG1 high-responders", the remaining 30% were "mIgG1 low-responders" (7,8). We and others reported that PBMC from normal individuals also exhibit polymorphism in their mitogenic response to mIgG2b anti-CD3 mAb (9-12). Among a group of 550 individuals, a mitogenic response to mIgG2b anti-CD3 mAb was observed in 3% ("mIgG2b high-responders") (13). In those individuals, the polymorphic receptor for mIgG2b was present on monocytes but also on other mononuclear cells that remained after thorough depletion of all monocytes from PBMC (13,14). Direct evidence for the presence of an Fc receptor for mIgG2b on (Epstein-Barr virus-transformed) human B cells was obtained by EA-rosetting (13).

Although the existence of a polymorphic human Fc receptor for mIgG2b was reported by several investigators as discussed above, and is undisputed, the molecular identity of this Fcγ receptor has remained elusive so far. This topic is confounded by the fact that several different Fc receptors can be present on one cell type. Human monocytes, for instance, exhibit mIgG2b-polymorphism in the T cell proliferation test (13), but monocytes from all donors (irrespective of responsiveness to mIgG2b anti-CD3) can perform ADCC against erythrocytes that have been highly sensitized with mIgG2b. Apparently, when polymeric mIgG2b is present at high concentrations, it can also bind to other monocyte Fc receptors that are not polymorphic with respect to mIgG2b (15). Although FcγRII may be able to bind polymeric mIgG2b under certain conditions (6), several findings argue against the hypothesis that (an isoform of) FcγRII is responsible for the polymorphic binding of mIgG2b. First, the

mIgG2b polymorphism is independent of the (FcγRII-mediated) polymorphism with respect to mIgG1: the percentage of mIgG2b high-responders is completely different from the percentage of mIgG1 high-responders, and the responder-status with respect to mIgG2b is independent of the responsiveness to mIgG1 (9). Furthermore, anti-FcγRII antibodies are not inhibitory for mIgG2b anti-CD3 induced T cell proliferation (13). Remarkably, and in contrast to the results obtained with mIgG1 anti-CD3, the mitogenic effect of mIgG2b anti-CD3 mAb (in mIgG2b high-responders) is not associated with release of interleukin-2 (IL2) or interferon-γ (IFNγ), and is not inhibited by anti-IL2 receptor antibody (16). Additional strong evidence for nonidentity of mIgG2b-receptor and FcγRII comes from experiments involving proteolytic enzymes. Proteolysis increases EA-rosetting mediated by mIgG2b, and at the same time decreases the expression of both FcγRII and FcεRII (17). Finally, the polymorphic receptor for mIgG2b is strongly inhibited by anti-HLA class II mAb, in an Fc-independent way. Such an apparent association between Fc receptor and HLA-DR is not found with FcγRI or FcγRII (18). Therefore, although the polymorphic mIgG2b receptor does not appear to be (an isoform of) FcγRII, its identity is still an open question that requires further investigations at the molecular level. Another unsolved question is how mIgG2b anti-CD3 mAb induces T cell proliferation in the absence of IL2. It is also not yet known how this Fc receptor for mIgG2b interacts with human IgG of different subclasses. In this respect it should be mentioned that the polymorphism of FcγRII is also physiologically relevant: mIgG1 low-responders exhibit an effective interaction with human IgG2 complexes whereas mIgG1 high-responders do not (19).

Clinical use of mIgG2b or mIgG1 antibodies

So in conclusion, polymeric mIgG2b can bind to a polymorphic Fc receptor in a small minority of normal individuals. In addition, it can bind to some extent (with a very low-affinity) to mononuclear cells from all individuals through Fc receptors that are not polymorphic with respect to mIgG2b. What are the implications of these interactions for the immunotherapeutical use of mIgG2b mAb? One would expect that in mIgG2b low-responders (the great majority of individuals), only target cells that are highly sensitized with mIgG2b would be able to interact with the nonpolymorphic binding sites present on monocytes or macrophages. Indeed, monocyte ADCC occurred with erythrocytes that were

sensitized with very high amounts of mIgG2b (approximately 3 x 10^5 antibody molecules per erythrocyte) (15), but not with tumour cells coated with a low number of mIgG2b antibody molecules (1). Consistent with this, administration of a mIgG2b mAb directed against the CD2 antigen on human T cells caused only coating in vivo of these T cells, and no elimination (and no immunosuppressive effect) (20). When the mIgG2b mAb BMA 031, directed against the CD3/ T cell receptor complex, was administered to renal transplant patients, there was only a partial and transient depletion of CD3-positive T cells. Furthermore, administration of this antibody was not accompanied by major side effects (21). This contrasts with the so-called first-dose response observed with mIgG2a anti-CD3 mAb OKT3 (and WT32). Fever, chills, and several other symptoms usually start within one hour after first administration of these mIgG2a antibodies. These symptoms are caused by cytokine release associated with (Fc receptor-dependent) T cell activation in vivo (22,23). BMA 031 does not induce IFNγ or IL2 in vitro, not even with mononuclear cells from mIgG2b high-responders (16), and after administration to renal allograft recipients it induces release of tumour necrosis factor but not of IFNγ or IL2. Apparently, synergism between cytokines is required to induce the characteristic clinical syndrome (24). Since BMA 031 is specific for the T cell receptor (in contrast to OKT3 that recognizes the CD3 antigen), it has been argued that the different results obtained with this antibody when compared to OKT3 are caused by its fine specificity rather than by its isotype. The pathways of T cell activation by BMA 031 or OKT3 are clearly different (12,25) but the relative contributions of isotype and specificity in this respect are not yet clear. Recently another mIgG2b mAb (BC3) was administered for treatment of graft-versus-host disease. This antibody, directed against a subunit of the CD3 antigen, also caused only mild side effects (26). This finding underlines the importance of the antibody isotype with respect to the adverse effects associated with anti-CD3/ T cell receptor antibodies. The exact binding segment in IgG molecules that determines the affinity for human Fc receptors has been defined. Using this information on the relevant amino acid sequence, the OKT3 antibody molecule has been modified in such a way that the FcR binding sequence became similar to the one found in mIgG2b. This modified OKT3 is still immunosuppressive in vitro, but does not cause T cell activation (due to its low-affinity for Fc receptors) and may be a good candidate to obtain effective immunosuppression without side effects (27).

What significance has the polymorphism of the Fc receptors for mIgG1 and mIgG2b in relation to the immunotherapeutical use of these isotypes? It has

been reported that elimination of CD8-positive T cells after administration of mIgG1 anti-CD8 mAb only occurred when the patients were high-responder with respect to mIgG1 (28). Our own preliminary data concerning WT31, a mIgG1 mAb directed against the T cell receptor, indicate that release of cytokines and clinical symptoms of "first-dose response" only occur with mIgG1 high-responders (Frenken et al., unpublished observations). To our knowledge, no mIgG2b high-responder individuals have yet been treated with a mIgG2b mAb. Since even with cells from mIgG2b high-responders, mIgG2b anti-CD3 mAb did not induce IL2 or IFNγ in vitro (as discussed above), we would expect that even in mIgG2b high-responder individuals such an antibody would not cause cytokine-related side effects. Still it is conceivable that mIgG2b high-responder patients respond in a different way to administration of mIgG2b mAb than mIgG2b low-responders. It has been reported, for instance, that antibody-induced modulation of an antigen from the cell surface can be enhanced by interaction of the antibody with an Fc receptor (29). Further clinical studies will be needed to define the relevance of Fc receptor polymorphism for the effectiveness and safety of mIgG1 or mIgG2b antibodies.

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Chapter 7

Summary

Summary

In the present thesis, the functional polymorphism of the human (h) Fc receptor (FcR) that interacts with murine (m) IgG2b antibodies was studied, and its relation with the currently known FcR was investigated. An overview of the human Fc receptors is given in **chapter 1**. Of the heterogeneous group of Fc receptors, predominantly hFcγRI, hFcγRII and hFcϵRII are expressed on the cells (monocytes and B cells) that are involved in Fc-mediated binding of mIgG2b antibodies. Therefore, these Fc receptors are described in some detail, and special attention is given to the previously reported polymorphism of hFcγRII. The polymorphism of this Fc receptor has been detected in a T cell proliferation assay. Anti-CD3 mAb can induce a mitogenic response of T cells, but this only occurs when the Fc part of the anti-CD3 mAb is able to interact with appropriate Fc receptors present on accessory cells. The mitogenic response to mIgG2a anti-CD3 mAb is mediated by monocyte hFcγRI, and occurs with peripheral blood mononuclear cells (PBMC) from virtually all individuals. On the contrary, a proliferative response of PBMC to mIgG1 anti-CD3 mAb (mediated by monocyte hFcγRII) only occurs in 70% of healthy individuals ("high-responders" with respect to mIgG1: mIgG1-HR), but not in the remaining 30% of mIgG1 "low-responders" (LR).

Anti-CD3 mAb of mIgG2b isotype is mitogenic for PBMC from less than 10% of normal individuals (mIgG2b-HR). In order to determine whether human monocytes and/or other cells in the mononuclear cell suspension are responsible for the accessory function in T cell proliferation induced by mIgG2b anti-CD3 mAb, a new method for depletion of monocytes was developed (**chapter 2**). This method involved panning with anti-CD14 mAb, and subsequent removal of any remaining monocytes with a magnet after phagocytosis of carbonyl-iron. The presence of (hFcγRI-positive) monocytes was measured in a mIgG2a anti-CD3 mAb induced T cell proliferation assay detecting less than 1% of monocytes. This method could be used with freshly isolated or cryopreserved mononuclear cells. Importantly, mIgG2b anti-CD3 mAb could still induce significant T cell proliferation after depletion of monocytes suggesting that other cells than monocytes (probably B cells) can also function as accessory cells in this model.

The functional polymorphism to mIgG1 anti-CD3 mAb (70% mIgG1-HR)

located on the hFcγRIIa1-encoded molecule expressed in monocytes was found to be independent from the mIgG2b-polymorphism. As described in **chapter 3**, high-responsiveness to mIgG2b anti-CD3 mAb was detected in 3% of the Caucasian individuals (mIgG2b-HR). Purified monocytes and (EBV-transformed) B cells derived from mIgG2b-HR were able to support mIgG2b anti-CD3 mAb induced mitogenic T cell responses. Anti-FcγRII mAb caused no inhibition of the mIgG2b anti-CD3 mAb induced mitogenic response. EBV-B cells from mIgG2b-HR could mediate EA-mIgG2b rosetting but no EA-mIgG1 rosetting was found. These findings indicate that the binding site for mIgG2b is different from the binding site for mIgG1, and may even be located on an Fc receptor different from hFcγRII.

Anti-HLA class II mAb completely inhibited the mIgG2b anti-CD3 mAb induced proliferative response as well as EA-mIgG2b rosetting (**chapter 4**). This inhibitory effect was Fc-independent since whole IgG as well as $F(ab')_2$ fragments thereof abolished the response. The FcR for mIgG2b was also present on HLA class II-negative EBV-B cells (from patients suffering from the "Bare Lymphocyte Syndrome"), which indicates that the FcR for mIgG2b is not identical to HLA class II, and does not require HLA class II molecules for its expression. In previous studies an association of HLA class II molecules with hFcεRII/CD23 on B cells had been reported. However, the involvement of hFcεRII in the binding of mIgG2b antibodies seemed to be unlikely because human IgE inhibited the mIgE anti-CD3 mAb induced T cell proliferation whereas no inhibition of the mIgG2b anti-CD3 mAb induced response was observed.

Additional evidence that the FcR for mIgG2b on B cells was different from hFcγRII or hFcεRII was obtained in studies on proteolysis (**chapter 5**). EBV-B cells from mIgG2b-HR individuals with normal HLA class II expression treated with exogenously added serine proteases (e.g. human leucocyte elastase or cathepsin G), showed an increase in EA-mIgG2b rosetting whereas the expression of both hFcγRII and hFcεRII was strongly reduced. Remarkably, proteolysis caused a reduced EA-mIgG2b rosetting in EBV-B cells from HLA class II-negative individuals. These data suggest that the Fc-binding epitope for mIgG2b in mIgG2b-HR individuals may be protected by HLA class II molecules against proteolytic damage.

The clinical significance of the polymorphic interaction of mIgG2b antibodies with human Fc receptors is discussed in **chapter 6**. When anti-CD3 antibody

is administered for immunosuppression in transplant recipients, the Fc moiety of the monoclonal antibody may interact with appropriate Fc receptors and initiate release of cytokines which are responsible for the side effects in vivo. Such a first-dose effect is seen with mIgG2a anti-CD3 mAb in all individuals. Preliminary data indicate that for mIgG1 anti-CD3 mAb this effect only occurs in mIgG1-HR individuals. With respect to mIgG2b, the majority of individuals demonstrates low-affinity for mIgG2b antibodies. In vitro studies revealed that mIgG2b anti-CD3 mAb caused no release of cytokines, not even with mIgG2b-HR cells. Therefore, in vivo use of mIgG2b anti-CD3 antibodies might be promising in future studies on immunosuppression. Indeed, the administration of BMA 031, a mIgG2b (anti-T cell receptor) mAb was not associated with severe side effects.

The investigations summarized above do not provide a definitive answer to the question which human Fc receptor is responsible for the polymorphic binding of mIgG2b antibodies. However, they strongly suggest that the Fc receptor for mIgG2b is different from hFcγRI, hFcγRII, hFcγRIII or hFcϵRII, for the following reasons:

- EBV-transformed B cells from mIgG2b-HR individuals can mediate EA-mIgG2b rosetting, and can support the mitogenic effect of mIgG2b anti-CD3 mAb, but they do not express hFcγRI or hFcγRIII (chapter 3).

- The hFcγRII is expressed on monocytes and B cells, and several isoforms of this receptor have been described. It is very unlikely, however, that hFcγRII is responsible for the polymorphic interaction with mIgG2b. Anti-FcγRII mAb do not inhibit mIgG2b anti-CD3 mAb induced T cell proliferation (chapter 3). A second, and probably even more convincing, argument is that proteolytic enzymes increase EA-mIgG2b rosetting and at the same time drastically decrease the expression of hFcγRII (chapter 5).

- The same argument holds with respect to hFcϵRII (chapter 5). Furthermore, the mitogenic response to mIgG2b anti-CD3 mAb is not inhibited by human IgE (chapter 4).

In future studies a characterization of the molecular structure of the Fc receptor that interacts with murine IgG2b may provide the molecular basis of the functional polymorphism described in the present study.

Samenvatting

Samenvatting

In dit proefschrift is een funktioneel polymorfisme beschreven van een humane (h) Fc receptor (FcR), die een interaktie kan aangaan met IgG2b antistoffen afkomstig van de muis (mIgG2b). De relatie van deze receptor met de tot nu toe bekende Fc receptoren is onderzocht. Een overzicht van de humane Fc receptoren is gegeven in **hoofdstuk 1**. Van een heterogene groep van Fc receptoren worden voornamelijk hFcγRI, hFcγRII en hFcεRII geëxprimeerd op de cellen (monocyten en B lymfocyten) die betrokken zijn bij de Fc-gemediëerde binding van muize IgG2b antistoffen. Deze Fc receptoren zijn dan ook uitgebreider beschreven waarbij speciale aandacht is geschonken aan het al eerder gevonden polymorfisme met betrekking tot de humane FcγRII. Het polymorfisme van deze Fc receptor werd voor het eerst beschreven in een T cel proliferatie-test. Anti-CD3 monoclonale antistoffen zijn in staat een mitogene T cel respons te induceren, maar dit vindt alleen plaats wanneer het Fc-gedeelte van de anti-CD3 antistof een interactie kan aangaan met geschikte Fc-receptoren op ondersteunende cellen. De mitogene respons op mIgG2a anti-CD3 antistoffen wordt gemediëerd door hFcγRI, en is gevonden met perifere bloed mononucleaire cellen (PBMC) van vrijwel alle individuen. Daarentegen vindt een proliferatieve respons van PBMC in aanwezigheid van mIgG1 anti-CD3 antistoffen (gemediëerd door hFcγRII op monocyten) plaats bij 70% van de gezonde individuen ('high-responders' met betrekking tot mIgG1: mIgG1-HR), maar niet bij de resterende 30% mIgG1 'low-responders' (LR).

De anti-CD3 antistof van het mIgG2b isotype is mitogeen in PBMC van minder dan 10% van de normale individuen (mIgG2b-HR). Om te bepalen of humane monocyten en/of andere cellen in de mononucleaire celsuspensie verantwoordelijk zijn voor de ondersteunende funktie in de T cel proliferatie geïnduceerd door mIgG2b anti-CD3 antistoffen, is een nieuwe methode ontwikkeld om monocyten te elimineren (**hoofdstuk 2**). Deze methode omvat eerst 'panning' met anti-CD14 antistoffen waarbij vervolgens de nog resterende monocyten, na opname van carbonyl-ijzer werden verwijderd met een magneet. De aanwezigheid van (hFcγRI-positieve) monocyten werd gemeten in een mIgG2a anti-CD3 antistof geïnduceerde T cel proliferatie-test waarbij de aanwezigheid van minder dan 1% monocyten nog gedetecteerd kon worden. Deze methode is bruikbaar voor zowel vers geïsoleerde als in vloeibare stikstof bewaarde mononucleaire cellen. Belangrijk is, dat na het verwijderen van de

monocyten de mIgG2b anti-CD3 antistof geïnduceerde T cel proliferatie nog steeds gemeten kon worden. Dit veronderstelt dat andere cellen dan monocyten (waarschijnlijk B lymfocyten) in staat zijn om als ondersteunende cel in dit model te fungeren.

Het funktionele polymorfisme voor mIgG1 anti-CD3 antistoffen (70% mIgG1-HR individuen) op monocyten gecodeerd door hFcγRIIa1, is onafhankelijk van het mIgG2b-polymorfisme. Zoals beschreven in **hoofdstuk 3**, is een sterke respons op mIgG2b anti-CD3 antistoffen waargenomen bij slechts 3% van de individuen (mIgG2b-HR). Gezuiverde monocyten en (met EBV-getransformeerde) B lymfocyten afkomstig van mIgG2b-HR, zijn in staat om de mIgG2b anti-CD3 antistof geïnduceerde mitogene T cel respons te ondersteunen. Anti-FcγRII antistoffen veroorzaken geen remming van deze mitogene respons. EBV-B lymfocyten van mIgG2b-HR kunnen wel de EA-mIgG2b rozettering mediëren maar er werd geen EA-mIgG1 rozettering gevonden. Deze gegevens wijzen erop, dat de bindingsplaats voor mIgG2b verschillend is van de bindingsplaats voor mIgG1, of zelfs gelokaliseerd kan zijn op een Fc receptor die een andere is dan hFcγRII.

Antistoffen gericht tegen HLA klasse II antigenen geven volledige remming van zowel de mIgG2b anti-CD3 antistof geïnduceerde T cel proliferatie alsmede de EA-mIgG2b rozettering (**hoofdstuk 4**). Dit remmende effect is onafhankelijk van het Fc-gedeelte van het IgG omdat zowel het complete IgG als de $F(ab')_2$ fragmenten ervan de respons volledig remmen. De Fc receptor voor mIgG2b is ook gevonden op HLA klasse II-negatieve EBV-B lymfocyten (van patiënten die lijden aan het 'Bare Lymphocyte Syndrome'). Dit betekent dat de Fc receptor voor mIgG2b niet identiek is aan HLA klasse II, en dat HLA klasse II molekulen ook niet nodig zijn voor de expressie deze Fc receptor. In voorgaande studies is een associatie op B lymfocyten beschreven van HLA klasse II molekulen met hFcεRII/CD23. Echter, de betrokkenheid van hFcεRII bij de binding van mIgG2b antistoffen lijkt niet aannemelijk omdat humaan IgE wel de mIgE anti-CD3 antistof geïnduceerde T cel proliferatie remt, maar geen inhibitie geeft van de mIgG2b anti-CD3 antistof geïnduceerde respons.

Aanvullende bewijzen dat de Fc receptor voor mIgG2b op B lymfocyten verschillend is van hFcγRII of hFcεRII, zijn verkregen in studies naar effecten van proteolyse (**hoofdstuk 5**). EBV-B lymfocyten van mIgG2b-HR individuen met een normale HLA klasse II expressie en behandeld met exogeen toegevoegde serine proteases (b.v. humaan leucocyt elastase en cathepsine G),

vertonen een verhoogde EA-mIgG2b rozettering terwijl de expressie van zowel hFcγRII als hFcεRII sterk gereduceerd is. Opmerkelijk is, dat met EBV-B lymfocyten van HLA klasse II-negatieve individuen na proteolyse, een verlaagde EA-mIgG2b rozettering gevonden werd. Deze gegevens suggereren dat het Fc-bindende epitoop voor mIgG2b in mIgG2b-HR individuen door HLA klasse II moleculen beschermd wordt tegen proteolytische schade.

De klinische betekenis van de polymorfe interaktie van mIgG2b antistoffen met humane Fc receptoren wordt besproken in **hoofdstuk 6**. Wanneer ter bestrijding of voorkoming van afstoting anti-CD3 antistoffen toegediend worden aan een ontvanger van een transplantaat, is het mogelijk dat het Fc-gedeelte van de monoclonale antistof een interaktie kan aangaan met een geschikte Fc receptor waarbij cytokines kunnen vrijkomen, die verantwoordelijk zijn voor de bijwerkingen die in vivo worden gevonden. Dit "eerste dosis-effect" wordt na toediening van mIgG2a anti-CD3 antistoffen gevonden bij alle individuen. Voorlopige gegevens met mIgG1 anti-CD3 antistoffen duiden erop, dat dit effect alleen voorkomt bij mIgG1-HR individuen. Met betrekking tot mIgG2b is gebleken, dat het merendeel van de individuen een lage affiniteit voor mIgG2b antistoffen heeft. In vitro studies hebben uitgewezen dat bij gebruik van mIgG2b anti-CD3 antistoffen geen cytokines vrijkomen, ook niet door cellen van mIgG2b-HR. Gebruik, in vivo, van mIgG2b anti-CD3 antistoffen zou daarom veelbelovend kunnen zijn in toekomstige studies op het gebied van de immuunsuppressie. Inderdaad ging de toediening van BMA 031, een mIgG2b (anti-T cel receptor) antistof niet gepaard met sterke bijwerkingen.

Het onderzoek zoals hierboven samengevat, geeft geen definitief antwoord op de vraag welke humane Fc receptor verantwoordelijk is voor de polymorfe binding van mIgG2b antistoffen. De Fc receptor voor mIgG2b lijkt in ieder geval verschillend te zijn van hFcγRI, hFcγRII, hFcγRIII of hFcεRII, om de volgende redenen:

- EBV-getransformeerde B lymfocyten van mIgG2b-HR individuen kunnen de EA-mIgG2b rozettering mediëren en kunnen het mitogene effect van mIgG2b anti-CD3 antistoffen ondersteunen, maar zij exprimeren hFcγRI of hFcγRIII niet (hoofdstuk 3).

- hFcγRII wordt tot expressie gebracht op monocyten en B lymfocyten. Verschillende isovormen van deze receptor zijn beschreven. Het is echter niet

waarschijnlijk dat hFcγRII verantwoordelijk is voor de polymorfe interaktie met mIgG2b. Anti-FcγRII antistoffen remmen immers de mIgG2b anti-CD3 antistof geïnduceerde T cel proliferatie niet (hoofdstuk 3). Een tweede, en waarschijnlijk meer overtuigend argument is dat behandeling met proteolytische enzymen de EA-mIgG2b rozettering verhoogt maar tegelijkertijd een drastische verlaging van de expressie van hFcγRII teweegbrengt (hoofdstuk 5).

- Hetzelfde argument geldt ook met betrekking tot hFcεRII (hoofdstuk 5). Bovendien wordt de mitogene respons van mIgG2b anti-CD3 antistoffen niet geremd in aanwezigheid van humaan IgE (hoofdstuk 4).

In toekomstige studies zal een karakterisering van de moleculaire struktuur van de humane Fc receptor die een interaktie aangaat met mIgG2b, mogelijk opheldering verschaffen over de moleculaire achtergronden van het funktionele polymorfisme zoals beschreven in deze studie.

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